

# **Microbial Transformations of Nitro- and Cyano-arenes.**

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*For my parents,  
Jamila and Rafiq*



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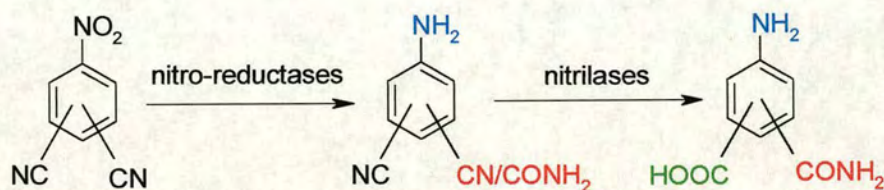
## Aims.

The initial aims of this project were several-fold. First, to investigate the scope of *Saccharomyces cerevisiae* catalysed nitroarene reduction in (di)cyanonitroarenes and to further probe the substrate specificity particularly with respect to regioselective and enantioselective transformations in (di)nitroarene sulfinyl substrates.

Second, to elucidate the mechanism of enzyme-catalysed nitro group reduction by the introduction of isotopically labelled substrates and to determine the intermediates involved in this pathway.

Third, to identify and characterise the nitro-reductase enzymes presents in *S. cerevisiae*, which catalyse the reduction of nitroarenes.

Finally, to carry out hydrolysis of (di)cyanonitroarenes using another whole cell microorganism (*Rhodococcus* species) and to further investigate the combination of nitro-reductases and nitrilases to provide access to a wide range of functionalised aromatic compounds.



Biocatalytic reduction and hydrolysis of (di)cyanonitroarenes.



## Abstract.

*Saccharomyces cerevisiae* is known to reduce aromatic nitro groups to the corresponding anilines in a range of compounds<sup>1-7</sup>, but the mechanism employed by this whole-cell microorganism remains largely unexplored. We have reported on the reduction of a range of (di)cyanonitroarene compounds by *S. cerevisiae* in aqueous media, at neutral pH at room temperature. The work was extended to determine the stereoselectivity of *S. cerevisiae* towards racemic (di)nitroarene-sulfinyls. The sulfinyls were excellent substrates for the *S. cerevisiae* catalysed reduction but the reactions proceeded with little or no enantioselectivity and/or regioselectivity.

Mechanistic studies of the biotransformations of dicyanonitroarene substrates suggest that heterocyclic intermediates are involved in this reduction process. A mechanism has been proposed and by carrying out isotopic labelling studies, steps have been taken towards the elucidation of the intermediate(s) involved in the reduction process catalysed by *S. cerevisiae*.

3 Nitro-reductases isolated from *S. cerevisiae* have been studied with respect to their ability to reduce the nitro group in simple nitro aromatic compounds.

Finally, the hydrolysis of cyano groups in (di)cyanonitroarenes by *Rhodococcus* species containing a nitrilase has been investigated. The reduction and hydrolysis of (di)cyanonitroarenes using nitro-reductases and nitrilases respectively has been combined to obtain highly functionalised aromatic compounds.



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Finally, last but not least, my Mum, Dad and Family for their support throughout the three years and always being there. I love you and Thank you.



## Abbreviations.

There follows a list of common abbreviations used in the text of this thesis.

Ac	Acetyl
Bn	Benzyl
Bp	Boiling point
br	Broad
Bu	Butyl
CDCl <sub>3</sub>	Deuteriochloroform
CHCl <sub>3</sub>	Chloroform
CI	Chemical ionisation
Da	Daltons
D	Doublet
Dd	Doublet of doublets
$\delta_{\text{H}}$	Chemical shift of proton (NMR)
$\delta_{\text{C}}$	Chemical shift of carbon (NMR)
D	Day
d.e.	Diastereomeric excess
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
e.e.	Enantiomeric excess
EI	Electron impact ionisation
eq.	Equivalent
ES	Electrospray
Et	Ethyl
EtOAc	Ethyl acetate
EtOH	Ethanol
FAB	Fast atom bombardment
GCMS	Gas chromatography mass spectrometry
h	Hour



HPLC	High performance liquid chromatography
$J$	Spin coupling constant
LCMS	Liquid chromatography mass spectrometry
Me	Methyl
MeCN	Acetonitrile
min	Minute
Mp	Melting point
m	Multiplet
$m/z$	Mass to charge ratio
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NMR	Nuclear magnetic resonance
nOe	Nuclear Overhauser effect
Ph	Phenyl
Pr	Propyl
$R_f$	Retention factor (TLC)
$R_t$	Retention Time (HPLC/GCMS/LCMS)
s	Singlet
sept	Septet
sat.	Saturated
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
sp.	Specie
spp.	Species
t	Triplet
td	Triplet of doublets
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
TLC	Thin layer chromatography
UV	Ultra-violet
v/v	Volume to volume ratio



## 1. Introduction.

### 1.1 *Saccharomyces cerevisiae* : General.<sup>8,9</sup>

*Saccharomyces cerevisiae* (*S. cerevisiae*) is a yeast that is rich in intra- and extra-cellular enzymes. This microorganism, commonly known as baker's yeast, is widely used in synthetic organic chemistry, primarily because it is inexpensive and readily available. The yeast is available in freeze-dried form and hence is ideally suited for non-microbiologists since it does not require sterile conditions and can be handled using standard laboratory equipment and techniques.

*S. cerevisiae* can be used in three forms:

(a) Resting cells: The commercially available freeze-dried cells can be activated by resuspension in water and buffer and stored in a flask before the substrate is added. The main advantage of resting cells is that the reactions are cleaner and simpler and the method is generally applicable for laboratory-scale preparations. Co-factor regeneration occurs at the expense of endogenous carbohydrate stored in cells of *S. cerevisiae* leading to facile product recovery.

(b) Fermenting conditions: (i) use of carbohydrates (glucose or sucrose) as energy sources. Reaction times can range from several hours to several days with further addition of *S. cerevisiae* and/or carbohydrate at fixed time intervals. The carbohydrates are used to regenerate the NAD(P)H which is necessary for the reduction.<sup>10</sup>

(b) Fermenting conditions: (ii) ethanol can be used as a substitute for carbohydrates in *S. cerevisiae* reduction. The reaction in the presence of ethanol is much cleaner and does not form by-products.

(c) Immobilised cells: cells of *S. cerevisiae* can be absorbed or adhered onto a water-



insoluble support such as a commercial resin, agar or calcium alginate. An alternative is the crosslinking of the functional groups present on the surface of *S. cerevisiae* with the support. Application of this technique seems to offer little advantage over the above procedure, since yields obtained are usually lower.<sup>11</sup>

Use of organic solvents in *S. cerevisiae* biotransformations can be more advantageous over conventional water suspensions. Organic solvents can increase the conversion of poorly water soluble substrates or products by decreasing the substrate or product concentration in the pure water phase thus minimising the inhibitory effect and any undesired side reactions taking place during the biotransformation.

## 1.2 *S. cerevisiae* mediated biotransformations.<sup>10, 12-15</sup>

The majority of studies on the synthetic capability of *S. cerevisiae* deal with the reduction of carbonyl groups and C=C double bonds to afford a product which has one or more asymmetric carbon atoms. There are many examples of *S. cerevisiae* catalysed biotransformations in the literature and some of these reports are reviewed in the next section.

### 1.2.1 Hydrolysis of esters.

The hydrolysis of esters has only recently been a focus of thorough studies with esterases from *S. cerevisiae* shown to have hydrolytic activity.<sup>16</sup> Thus, esters of racemic mappicine acetate **1a**, **1b** were enantioselectively hydrolysed by fermenting *S. cerevisiae* to yield (*S*)-mappicine **2a**, **2b** in high optical purity in 48 % and 47 % yields respectively (Fig 1-1).<sup>17, 18</sup> The unchanged (*R*)-mappicine **3a**, **3b** acetate was hydrolysed chemically to afford the optically active (*R*)-mappicines in 48 % and



47 % yields respectively.

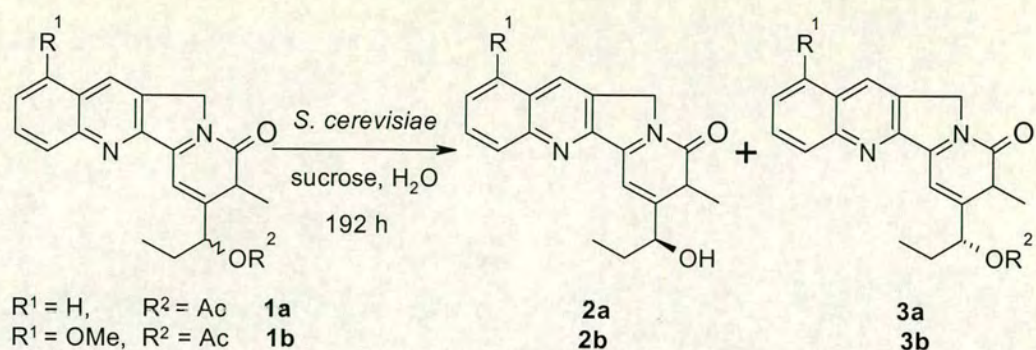


Fig 1-1 : *S. cerevisiae* catalysed hydrolysis of esters.

### 1.2.2 Hydrolysis of phosphates.

*S. cerevisiae* contains phosphatases which have been reported to selectively hydrolyse phosphates in positions 3, 4 and 5 of phytic acid **4** leading to the formation of an optically active inositol derivative **5** which exhibits anti-inflammatory properties (Fig 1-2).<sup>19</sup>

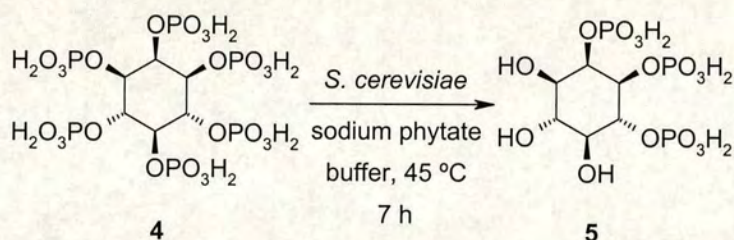


Fig 1-2 : *S. cerevisiae* catalysed hydrolysis of phosphate esters.

### 1.2.3 Formation of C-C bonds.

A recent example of a yeast catalysed acyloin condensation, which was first reported 60 years ago,<sup>20</sup> is the formation of alkylated cyanoketones **7**. The racemic alkylated derivatives **7** were obtained from **6** in moderate yields (59-75 %) in a two phase solvent system comprising of petrol and water (Fig 1-3).<sup>21</sup>



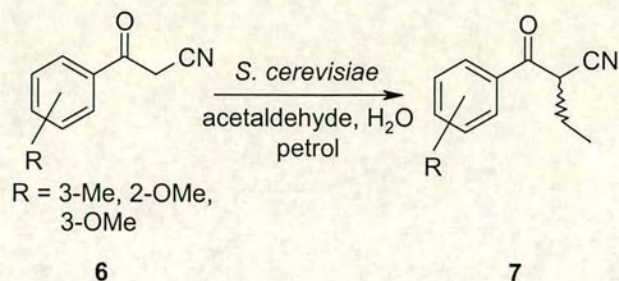


Fig 1-3 : *S. cerevisiae* catalysed C-C bond formation in aliphatic compounds.

Fuganti *et al.* have proposed a mechanism for this alkylation reaction using ethyl cyanoacetate **8** as the substrate (Fig 1-4).<sup>22</sup>

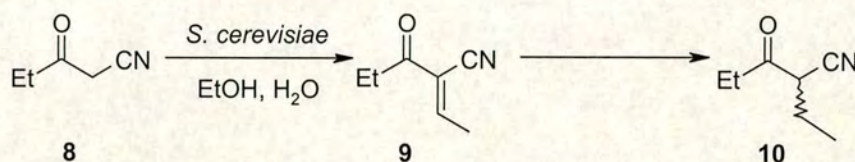


Fig 1-4 : Proposed mechanism for *S. cerevisiae* catalysed C-C bond formation.

The first step was believed to be the *S. cerevisiae* oxidation of ethanol to acetaldehyde. Condensation between the aldehyde and the active methylene group of the nitrile **8** afforded the  $\alpha$ - $\beta$ -unsaturated ketone **9** and finally a double bond reduction afforded the racemic product **10**.

#### 1.2.4 Oxidation.

The oxidative capabilities of *S. cerevisiae* are limited and hence literature examples of oxidations carried out by *S. cerevisiae* are scarce. A recent paper described the Baeyer-Villiger oxidation of substituted cyclic lactones **11** using recombinant *S. cerevisiae*.<sup>23</sup> The reaction was carried out by a cyclohexanone monooxygenase, which was cloned from the bacterium *Acinetobacter* sp. NCIMB 9871 and over-expressed in *S. cerevisiae* (Fig1-5).



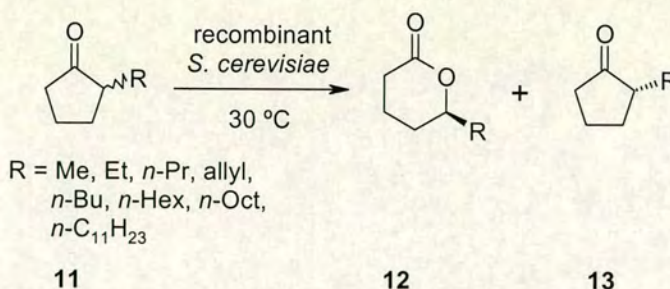


Fig 1-5 : *S. cerevisiae* catalysed oxidation of 2-substituted cyclopentanones.

The best results were obtained for compounds possessing long alkyl chains in the 2-position to afford the (*S*)-lactone **12** with e.e. of 98 %, whereas the other enantiomer **13** remained intact. Very low e.e.'s were obtained for lactone products obtained from 3-substituted pentanones.

Oxidation of alkyl-substituted cyclohexanones **14** have also been reported to yield the corresponding lactones **15** with good purities and e.e. greater than 92 %, whereas the other enantiomer **16** remained intact (Fig 1-6).<sup>24</sup>

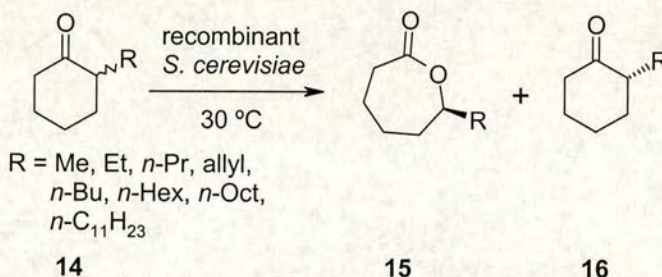


Fig 1-6 : *S. cerevisiae* catalysed oxidation of 2-substituted cyclohexanones.

Overall, cyclohexanone derivatives **14** were oxidised more efficiently compared to their cyclopentanone **11** counterparts.

Cyclic sulfoxides, which are important chiral intermediates in organic synthesis have been synthesised by the oxidation of cyclic dithiolanes **17** and dithianes **18** mediated by *S. cerevisiae* (Fig 1-7).<sup>25</sup> The corresponding sulfoxides **19** were obtained in lower optical purity and a higher proportion of the corresponding sulfone **20** was produced.



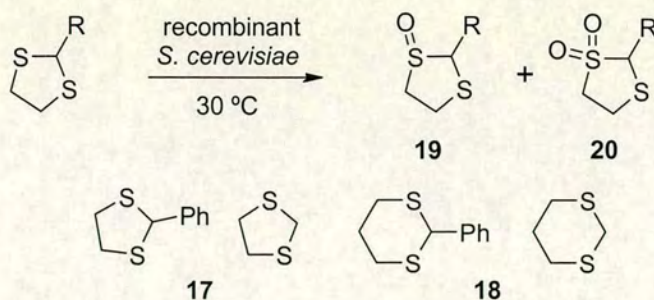


Fig 1-7 : *S. cerevisiae* catalysed oxidation of dithiolanes and dithianes.

### 1.2.5 Reductions.

Reduction reactions have constituted the major part of *S. cerevisiae* mediated transformations. Most simple aromatic and aliphatic compounds **21** are reduced by *S. cerevisiae* according to Prelog's rule<sup>26</sup> to afford the corresponding (*S*)-alcohols **22** in good optical purity (Fig 1-8), although exceptions to the stereospecificity are known: long chain ketones and several bulky phenyl carbonyl compounds do not follow this rule.

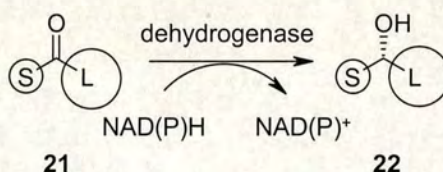


Fig 1-8 : Prelog's rule: Model representing reduction of the carbonyl moiety.

Typical substrates include:

- $\beta$ -Keto esters: aromatic, cyclic, heterocyclic, halogen and aliphatic.
- $\alpha$ -Keto esters: cyclic and aliphatic.
- $\gamma$ -Keto esters and acids.
- $\delta$ -Keto esters and acids.
- Diketones: cyclic, heterocyclic and acyclic.
- Monocarbonyl compounds: cyclic, aliphatic, heterocyclic, polycyclic, sulfur, nitro and hydroxy.
- C=C double bond reduction: cyclic and aliphatic.
- $\alpha$ - $\beta$ -Unsaturated enone systems: cyclic, aliphatic and halogen.



1.2.5.1  $\beta$ -Keto ester reduction.

Although the reactions have been known and exploited for a very long time, *S. cerevisiae* catalysed reduction of  $\beta$ -keto esters to afford optically active  $\beta$ -hydroxy esters is still a very active field of research owing to the numerous synthetic applications of the synthons produced.

The enantioselective reduction of ethyl 3-oxobutanoate **23** mediated by *S. cerevisiae* has been studied extensively to afford the optically active hydroxy ester (*S*)-**24**.<sup>27</sup> Whilst the yields were only moderate (50-70 %), the reduction proceeded with excellent e.e. of 99.5 % (Fig 1-9).<sup>28</sup>

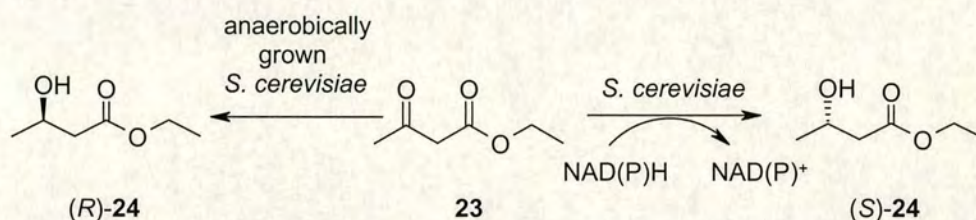


Fig 1-9 : *S. cerevisiae* catalysed reduction of ethyl 3-oxobutanoate.

Dahl *et al.* have shown the selectivity of the above reduction can be reversed using anaerobically grown *S. cerevisiae* to afford the alcohol (*R*)-**24** with an e.e. of 96 %.<sup>29</sup>

*S. cerevisiae* may still be the catalyst of choice for the reduction of some cyclic keto esters since optically pure diastereomeric hydroxy esters can be obtained (Fig 1-10).<sup>30</sup> The hydroxy ester **26** obtained in 40 % yield was a result of the kinetic resolution of the racemic starting material **25**.

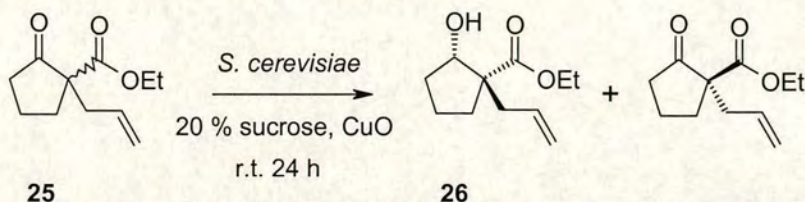


Fig 1-10 : *S. cerevisiae* catalysed reduction of cyclic  $\beta$ -keto ester.



The reduction of heteroatom substituted  $\beta$ -keto esters by *S. cerevisiae* has been reported. Enantiomerically pure  $\beta$ -hydroxy ester derivatives **28** possessing a sulfur heteroatom group were obtained in moderate yields and high e.e. of 92-99 % by the reduction of heterocyclic keto ester derivatives **27** (Fig 1-11).<sup>31</sup>

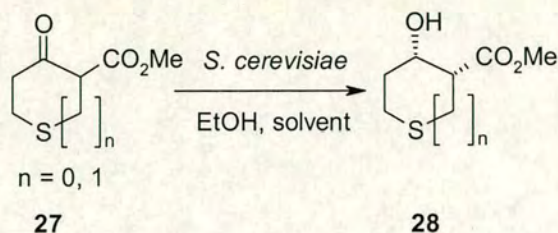


Fig 1-11 : *S. cerevisiae* catalysed reduction of heterocyclic  $\beta$ -keto ester.

Under non-fermenting conditions, the reduction of a cyclic  $\beta$ -keto ester **29** afforded an enantiomer of substituted 2-piperidone **30** in 88 % optical yield with e.e. of 91 % (Fig 1-12). These optically active hydroxy piperidine esters are present in many pharmaceutical and alkaloid compounds.<sup>32</sup>

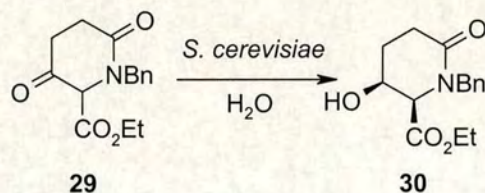


Fig 1-12 : *S. cerevisiae* reduction of a heterocyclic  $\beta$ -keto ester.

There have been reports on the selective inhibition of different enzymes with opposite specificity when different additives are used in the reduction of  $\beta$ -keto esters.<sup>33</sup> A recent report has described the effect of using allyl chloride or allyl alcohol as additives in the presence of *S. cerevisiae* to obtain both the (*R*)- and (*S*)-**32** enantiomers of trifluoro-3-oxo-butanoate **31** with e.e.'s in the range of 56-80 % (Fig 1-13).<sup>34</sup>



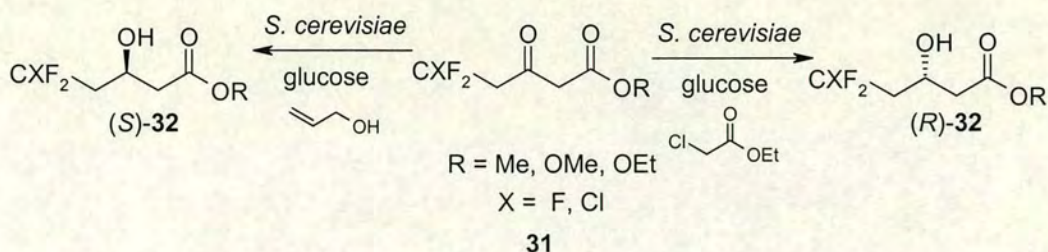


Fig 1-13 : *S. cerevisiae* catalysed reduction of halogen containing  $\beta$ -keto esters.

Another paper has reported on the synthesis of an acetal protected 4-amino-2-pentanone **35**, the key step being the reduction of an aliphatic 4,4-dimethoxybutan-2-one **33** to afford the optically active hydroxy derivative **34** in 75 % yield with e.e. of 98 % (Fig 1-14).<sup>35</sup>

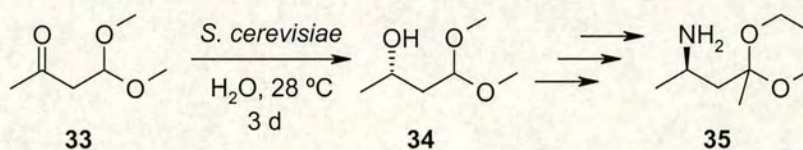


Fig 1-14 : *S. cerevisiae* catalysed reduction of  $\beta$ -keto compound.

$\alpha$ -Hydroxy- $\beta$ -keto esters are good substrates for stereospecific reduction catalysed by *S. cerevisiae* to yield the corresponding dihydroxy esters **37** with high e.e.'s ranging from 95-98 % (Fig 1-15).<sup>36</sup> Only one enantiomer of the racemic substrates **36** was reduced to the optically active *trans* dihydroxy compounds **37** effecting a resolution of the starting material.

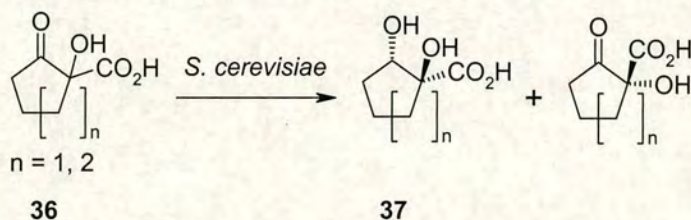


Fig 1-15 : *S. cerevisiae* catalysed reduction of cyclic  $\alpha$ -hydroxy- $\beta$ -keto esters.

The reduction of  $\alpha$ -substituted- $\beta$ -keto esters by *S. cerevisiae* was strongly dependent on the pH of the medium. Thus ethyl-2-hydroxy-3-oxo-3-phenylpropanoate ( $\text{R} = \text{OH}$ ) **38a** was reduced by *S. cerevisiae* immobilised on calcium alginate beads,



affording 2-3-dihydroxy-3-phenylpropanoate **39a** as the major product in 82 % yield at low pH (Fig 1-16).<sup>37</sup>

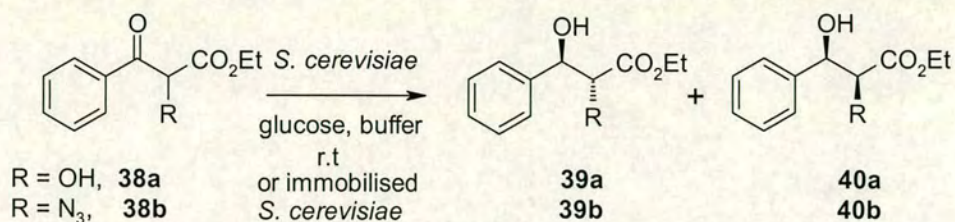


Fig 1-16 : *S. cerevisiae* catalysed reduction of cyclic  $\alpha$ -substituted- $\beta$ -keto esters.

Variation in the pH of the reaction resulted in a significant change in the diastereomeric and enantiomeric purity of the dihydroxy esters obtained. A higher ratio of 10:1 (**39a** and **40a**) respectively was observed at a low pH of 4, which gradually reached the expected value of 1:1 at pH 7.

The same effect was observed in the reduction of an  $\alpha$ -azido- $\beta$ -ketoester **38b** (R = N<sub>3</sub>) using immobilised *S. cerevisiae*. A higher ratio of 11:1 (**39b** and **40b**) respectively was observed at pH 4 (e.e. 99 %, d.e. 79 %).<sup>38</sup> Whilst the stereospecificity of the reduction was maintained, a loss of diastereoselectivity occurred at pH 7 where a 1:1 diastereomeric mixture was obtained.

#### 1.2.5.2 $\alpha$ -Keto ester reduction.

$\alpha$ -Keto esters have been successfully reduced by *S. cerevisiae* to  $\alpha$ -hydroxy esters, a simple example being the reduction of substituted  $\alpha$ -keto esters **41** to afford the optically active (*R*)-alcohols **42** with high e.e. in the range of 82-92 % (Fig 1-17).<sup>39</sup>



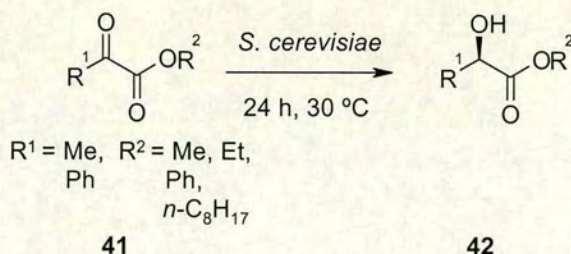


Fig 1-17 : *S. cerevisiae* catalysed reduction of aliphatic  $\alpha$ -keto esters.

A stereoselective synthesis of  $\alpha$ -hydroxy- $\beta$ -lactams **44** mediated by the microbial reduction of azetidin-2-3-dione **43** has recently been carried out using fermenting *S. cerevisiae* in water with the reduction proceeding slowly in 57 % yield with e.e. of 66 % (Fig.1-18).<sup>40</sup>

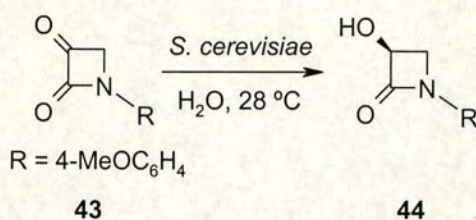


Fig 1-18 : *S. cerevisiae* catalysed reduction of  $\alpha$ -keto- $\beta$ -lactams.

### 1.2.5.3 $\gamma$ -Keto ester reduction.

*S. cerevisiae* catalysed reduction of cyclic  $\gamma$ -keto esters **45** proceeded with enantio-, diastereo-selectivity and simultaneous kinetic resolution affording the corresponding optically active cyclic *trans*-alcohols **46** (41 % and 48 % respectively) as the major product, *cis*-alcohols **46** (16 % and 19 % respectively) as the minor and the unaltered ketone **47** with optical purity of 98 % (Fig 1-19).<sup>41</sup>

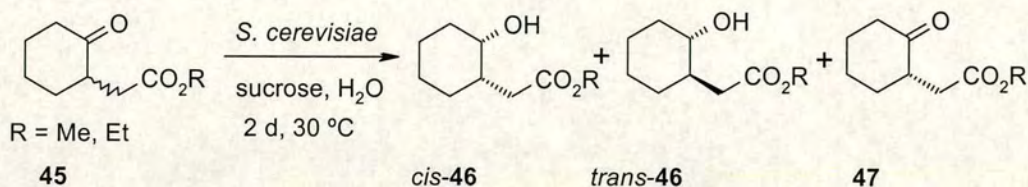


Fig 1-19 : *S. cerevisiae* catalysed reduction of cyclic  $\gamma$ -keto ester .



The optically enriched enantiomer of the  $\gamma$ -keto ester **47** can be employed as a precursor in the synthesis of lipoic acid, which is a growth factor for a variety of microorganisms.<sup>42</sup>

*S. cerevisiae* catalysed reduction of aliphatic  $\beta$ -chloro- $\gamma$ -keto esters **48** afforded the corresponding hydroxy product **49** and in some cases the  $\gamma$ -lactone **50** was obtained as a minor product (Fig 1-20).<sup>43</sup>

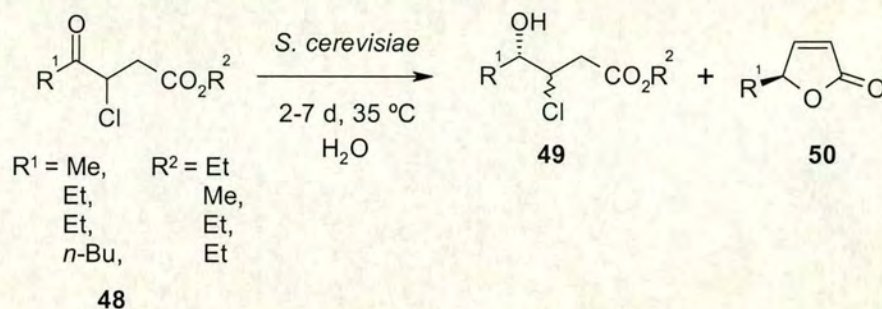


Fig 1-20 : *S. cerevisiae* catalysed reduction of aliphatic alkanooates.

Alkanooates with small alkyl groups ( $R^1 = \text{Me, Et}$ ) were reduced to the chiral alcohols **49** in good yields, whereas longer chain (butyl, octyl) compounds afforded lower yields of the reduced product **49** with formation of the lactone **50** as a minor impurity.

#### 1.2.5.4 $\delta$ -Keto ester reduction.

The formation of enantiopure  $\delta$ -lactones condensed with alicyclic rings has been achieved by the reduction of the corresponding  $\delta$ -keto esters **51a** and acids **51b** by *S. cerevisiae* with e.e and d.e greater than 99 % (Fig 1-21).<sup>44</sup>

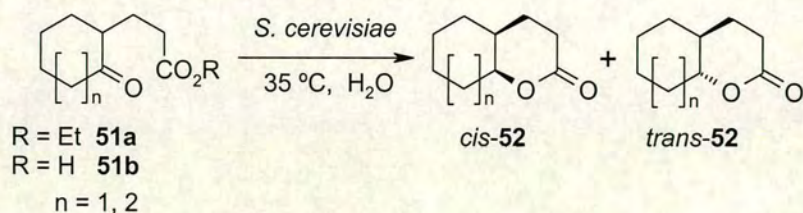


Fig 1-21 : *S. cerevisiae* catalysed reduction of aliphatic  $\delta$ -keto esters and acids.



The reduction proceeded with excellent stereospecificity when  $n = 1$ , forming only the *cis*-fused ring system **52** (86-99 %, e.e.) but was less diastereoselective for the larger ring size ( $n = 2$ ) in which case both lactones (*cis*- and *trans*-**52**) were formed. The keto acids **51b** ( $R = H$ ) proved to be worse substrates for the reduction than the keto esters **51a** with low yields obtained (5 %) with high diastereoselectivity (50 %).

#### 1.2.5.5 Diketone reduction.

A simple method involving the microbial reduction of  $\beta$ -diketones to prepare  $\beta$ -aminoketones has been achieved by the asymmetric reduction of 2,4-pentanedione ( $n = 1$ ) **53a** to the enantiomerically pure (*S*)-ketol **54** in 82 % yield with e.e. of 98 % (Fig 1-22).<sup>45</sup>

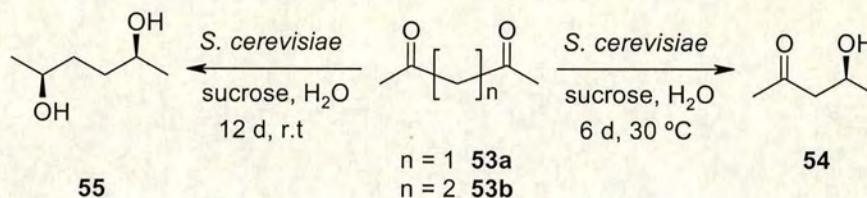


Fig 1-22 : *S. cerevisiae* catalysed reduction of aliphatic  $\beta$ -diketones.

In the case of acetonyl acetone ( $n = 2$ ) **53b**, the pure diol **55** was obtained with 59 % yield, d.e. and e.e. greater than 99 % (Fig 1-22).<sup>46</sup> A high concentration of substrate was used which improved both the reaction rate and selectivity with no traces of other stereoisomers formed.

A kinetic resolution of substituted bicyclic diketones under various conditions has been reported recently (Fig 1-23).<sup>47</sup>



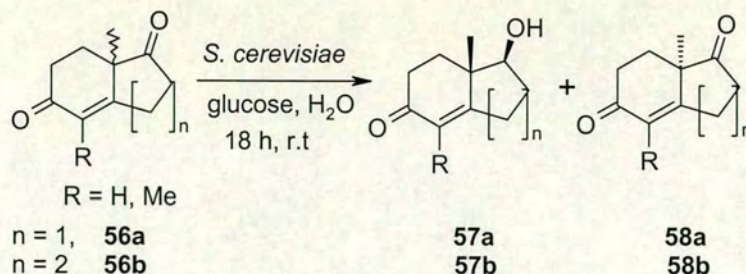


Fig 1-23 : *S. cerevisiae* catalysed reduction of bicyclic diketones.

After 24 h, the diketones ( $n = 1$ ) **56a** were reduced preferentially to the chiral alcohols **57a** in 54 % yield with e.e. greater than 72 %. A high concentration of substrate and *S. cerevisiae* was used which increased the enantioselectivity and the reaction rate leading to the recovery of enantiomerically pure diketone **58a**.

Expansion of the 5-membered ring by one carbon ( $n = 2$ ) **56b** resulted in the reduction proceeding much slower. Despite this, the enantiomerically pure alcohols **57b** were obtained in 32 % yield with e.e. of 99.5 %, along with the unreacted ketone **58b** in 60 % yield.

Other reducible groups present were tolerated in the synthesis of substituted spiroketals, which are found as substructures of many naturally occurring compounds present in plants and animals. The key step is the reduction of nitro-aliphatic diketones **59** to afford **60** which proceeded in moderate yields of 55 % and 67 % respectively while the nitro group remained intact (Fig 1-24).<sup>48</sup>

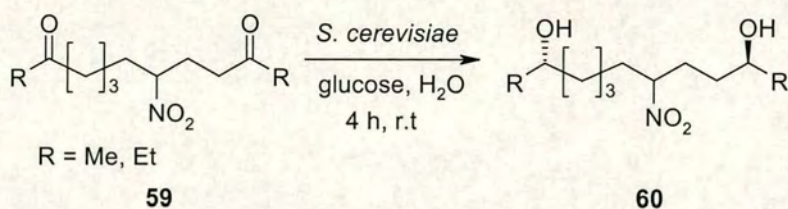


Fig 1-24 : *S. cerevisiae* catalysed reduction of nitro-diketones.



## 1.2.5.6 Carbonyl reduction.

The chemo- and stereo-selective reduction of a  $\beta$ -cyanoketone by *S. cerevisiae* at low temperature has been reported. The reduction of 3-oxo-3-phenylpropanenitrile derivatives **6** proceeded to afford exclusively the (*S*)-3-hydroxy-3-phenylpropanenitriles **62** in moderate yields with no alkylated product **7** produced at a low temperature of 4 °C (Fig 1-25).<sup>49</sup>

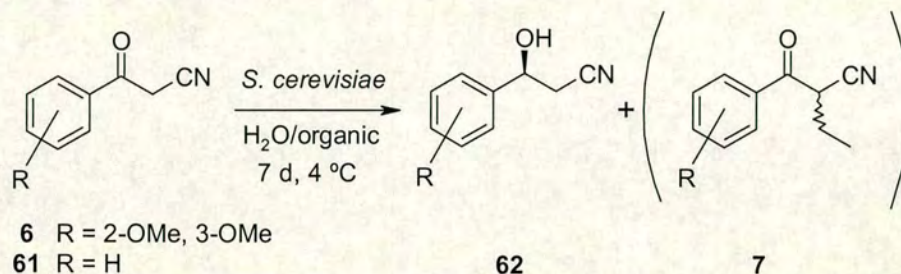


Fig 1-25 : *S. cerevisiae* catalysed reduction of aliphatic ketones.

With 3-oxo-3-phenylpropanenitrile **61**, both the reduced **62** and alkylated **7** products in 10 % and 40 % yields respectively were obtained when the reaction was carried out in a mixture of organic solvent and water at room temperature.<sup>21</sup>

Substituted aromatic rings **64** and **66** can be used in cancer chemotherapy and radiotherapy and the synthesis of these type of compounds involve a key step in which the reduction of 3-(hydroxyacetyl)-benzonitrile **63** affords the (*R*)-diol **64** in 44 % yield (Fig 1-26).<sup>50</sup> The optically active alcohol **65** with opposite stereochemistry was obtained in 85 % yield when the OH group was replaced with the OAc moiety.



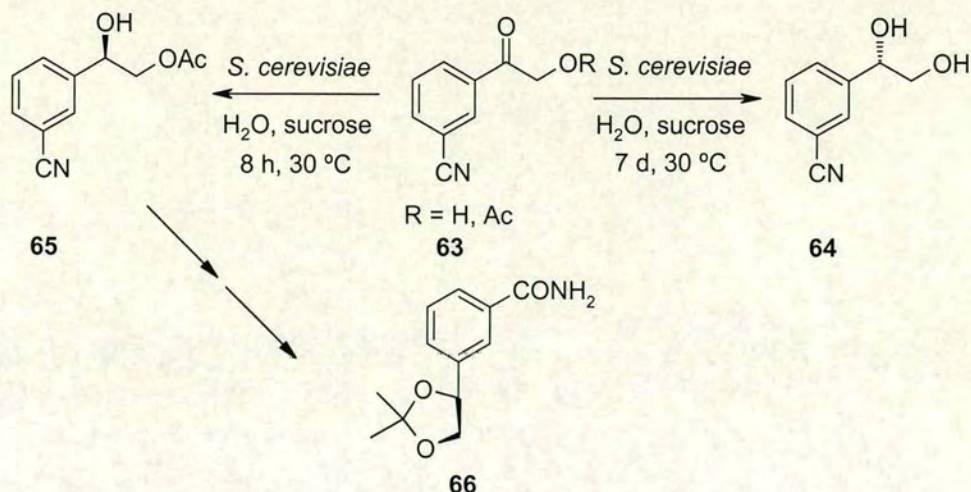


Fig 1-26 : *S. cerevisiae* catalysed reduction of  $\beta$ -hydroxy-ketone.

A mixture of concentrated yeast and sugar was used to reduce a series of methylsulfonylalkan-2-ones **67** with e.e. ranging between 71-87 % (Fig.1-27).<sup>51</sup>

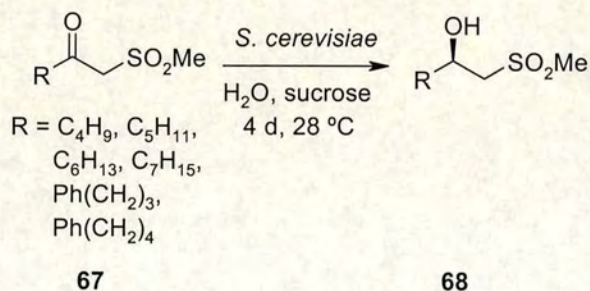


Fig 1-27 : *S. cerevisiae* catalysed reduction of aliphatic keto-sulfones.

The yields of (*S*)-alcohols **68** obtained, percentage conversions, e.e. and reproducibility were more satisfactory compared to those biotransformations carried out in dilute yeast and sugar mixtures. Using dilute conditions, (*R*)-alcohols were obtained for those substrates which had bulky substituents attached.

An efficient dynamic kinetic resolution of 5- and 6- membered cyclic  $\beta$ -keto sulfones **69** has been achieved using *S. cerevisiae* to afford the corresponding *cis*-benzene-sulfonylcycloalkanols **70** in d.e. and e.e. of 98 % and 95 % respectively (Fig 1-28).<sup>52</sup> The reduction of the 7- and 8-membered ring derivatives was much less efficient with very low enantiopurity obtained.



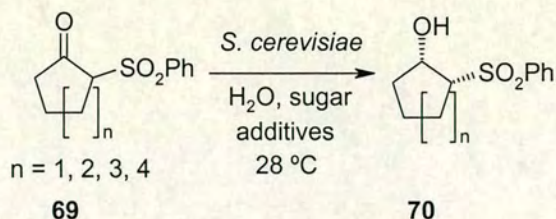


Fig 1-28 : *S. cerevisiae* catalysed reduction of cyclic keto-sulfones.

The reduction of heterocyclic ketones has been investigated where the ketones **71** are enantioselectively reduced to afford the corresponding (*S*)-alcohols **72** in 80-90 % yields with 100 % e.e. (Fig 1-29).<sup>53</sup>

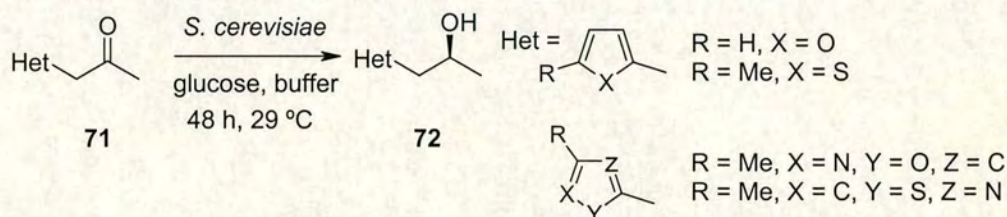


Fig 1-29 : *S. cerevisiae* catalysed reduction of heterocyclic ketones.

The asymmetric reduction of thiiranes **73** was found to be a useful method for the preparation of optically active  $\beta$ -hydroxy sulfides **74** with (*R*) configuration obtained in yields of 56-91 % with e.e. of 64-99 % (Fig.1-30).<sup>54, 55</sup>

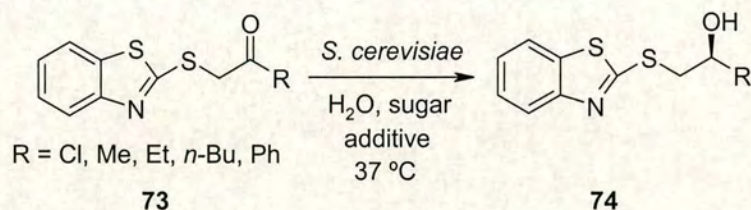


Fig 1-30 : *S. cerevisiae* catalysed reduction of heterocyclic ketones.

The reduced hydroxy sulfides **74** obtained could be further converted into the corresponding optically active amino-alcohols which display the molecular features of potential  $\beta$ -blockers used in cardiovascular disease.



There have been reports of *S. cerevisiae* biotransformations occurring in mixtures of water and organic solvents. The effect of changing the medium of the reaction on the enantioselectivity obtained has been investigated by Molinari *et al.*  $\gamma$ -Nitro ketone ( $n = 2$ ) **75b** was reduced to afford the chiral (*S*)-nitro alcohol **76b** with e.e of 89 % when buffer was used as the medium (Fig 1-31). The e.e was further increased to 95 % when a mixture of benzene and hexane was employed.<sup>56</sup> Similarly  $\beta$ -nitro ketone ( $n = 1$ ) **75a** in water was reduced to **76a** with 85 % e.e. and further increased to 97 % when a mixture of water and an organic solvent was used.

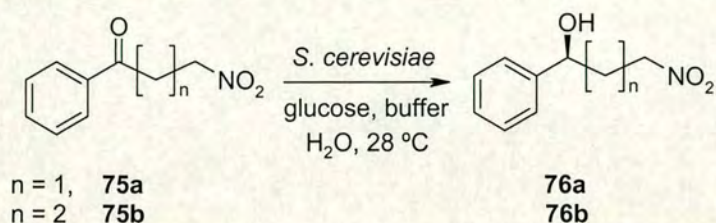


Fig 1-31 : *S. cerevisiae* catalysed reduction of  $\gamma$ - and  $\beta$ -nitro ketones.

The reduction of  $\pi$ -deficient heterocyclic aldehydes **77**, **78**, **79**, **80** can be catalysed by *S. cerevisiae* under fermenting conditions to afford chiral alcohols which are obtained by easy work-up in moderate yields of 50-83 % (Fig.1-32).<sup>57</sup>

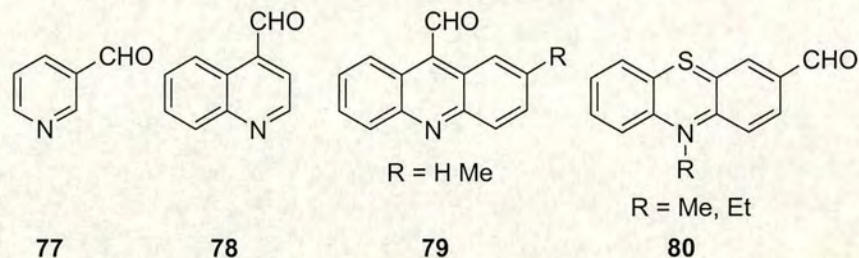


Fig 1-32 : *S. cerevisiae* catalysed reduction of heterocyclic aldehydes.

### 1.2.5.7 Enol and Enone reduction.

Fuganti *et al.* have reported on the reduction of unsaturated aldehydes **81** to afford diastereomerically enriched forms of saturated (*S*)-alcohols **82** in modest yields (51-54 %). The stereoselectivity of the reduction was greatly influenced by the E:Z ratio of the starting material (Fig 1-33).<sup>58</sup>



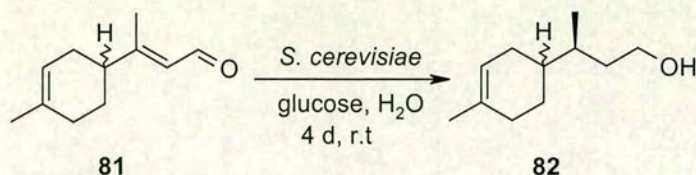


Fig 1-33 : *S. cerevisiae* catalysed reduction of  $\alpha$ - $\beta$ -unsaturated aldehydes.

The reduction of  $\alpha$ -bromo-substituted enone systems **83** to produce the enantiomerically pure 2-bromoindan-1-ol **84** in 75 % yield with e.e. of 99 % has been reported (Fig 1-34).<sup>59</sup>

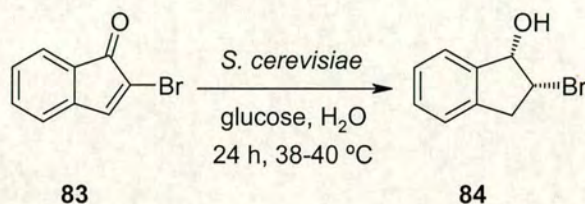


Fig 1-34 : *S. cerevisiae* catalysed reduction of cyclic enones.

Monitoring of the reaction indicated that the carbon-carbon double bond of **83** was reduced first to yield the ketone intermediate followed by reduction of the carbonyl moiety to produce the optically active saturated alcohol product **84**.

#### 1.2.5.8 C=C Double bond reduction.

*S. cerevisiae* displays chemoselectivity in thioacetals **85** which possess both a carbon-carbon double bond and a carbonyl moiety with the reduced ketones **86** obtained in 87 % e.e. in (*S*)-configuration (Fig 1-35).<sup>60</sup>

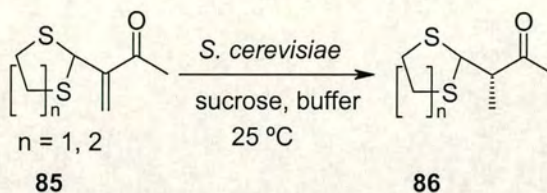


Fig 1-35 : *S. cerevisiae* catalysed reduction of aliphatic C=C.



The preparation of enantiomerically pure electron rich 3-arylinden-1-ones **88** with (*S*)-configuration has been reported with the reduction of **87** proceeding in moderate yields (50-81 %) with e.e. of 99 % (Fig 1-36).<sup>61</sup>

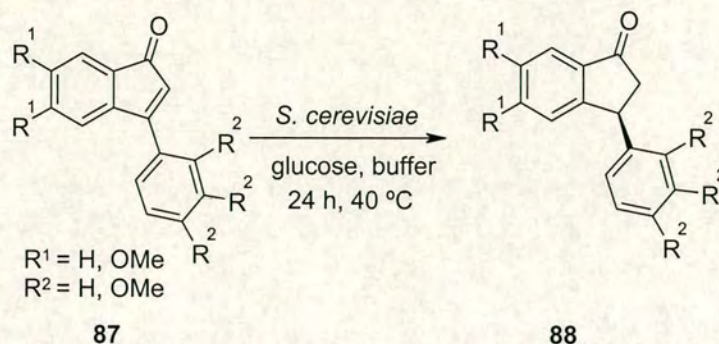


Fig 1-36 : *S. cerevisiae* catalysed reduction of cyclic C=C.

It was reported that changing the location of the electron-donating groups on either aromatic ring had little effect on the reaction outcome. The reduced product **88** was a key intermediate in the synthesis of a receptor-antagonist SB 217 42 which at present is in clinical trials at SmithKline Beecham Pharmaceuticals.

Parthenin **89**, an important terpenoid, when treated with *S. cerevisiae* underwent chemoselective reduction of the double bond(s) (Fig 1-37).<sup>62</sup> Both the double bonds were reduced to afford the saturated cyclic ketone **90** in 64 % yield with the carbonyl groups present remaining intact.

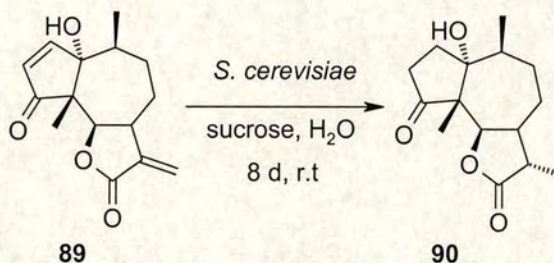


Fig 1-37 : *S. cerevisiae* catalysed chemoselective reduction of C=C.



## 1.2.5.9 Miscellaneous.

Ketoxime derivatives **91** are reduced to afford the corresponding optically active (*R*)-alcohols **92** in yields ranging from 48-75 % with excellent e.e. between 88-99 %. Two exceptions, where R = Me or H, afforded the reduced product **92** in poor e.e. and a low chemical yield of 14 % (Fig 1-38).<sup>63</sup> The low yield obtained was due to subsequent reactions in the medium to form the optically active diol **93**, whereas the other optically active oximes were relatively stable.

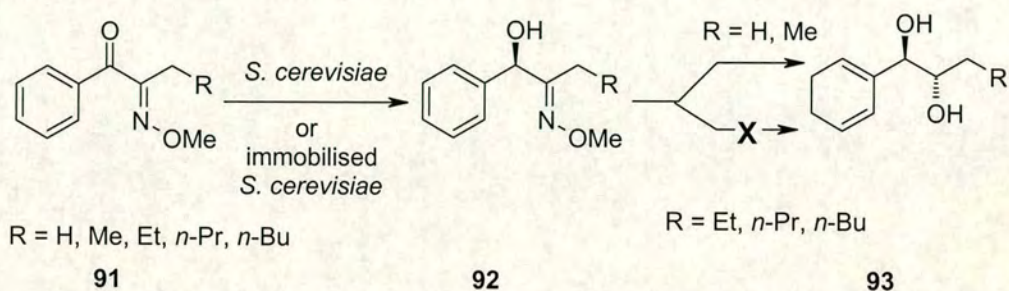


Fig 1-38 : *S. cerevisiae* catalysed reduction of ketoximes.

The reduction of the carbon-carbon triple bond in phenylbutyne **94** mediated by *S. cerevisiae* afforded the saturated compound **95** in a low yield of 7 % with the hydroxylated product **96** present as a minor impurity, hence displaying poor stereoselectivity (Fig 1-39).<sup>64</sup>

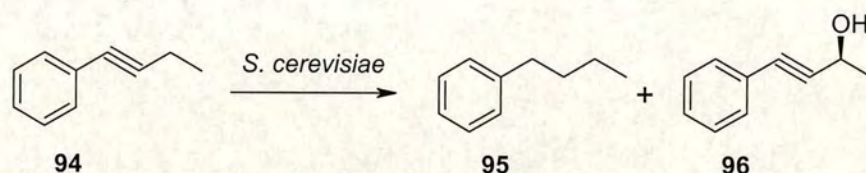


Fig 1-39 : *S. cerevisiae* catalysed reduction of  $\text{C}\equiv\text{C}$  triple bond.



### 1.3 *S. cerevisiae* mediated reduction of N-O containing groups.

Nitro groups are useful in synthetic chemistry because they can be easily converted into hydroxy, carbonyl and amino moieties which can be versatile synthetic intermediates. There have been a number of reports on the use of *S. cerevisiae* for the reductive cleavage of N-O bonds in a variety of functional classes including nitroarene, nitrosoarenes, nitroalkenes, *N*-oxides, isoxazoles, imines and azides and are discussed below.

#### 1.3.1 Nitroarenes.

Neuberg reported on the conversion of nitrobenzene to aniline using fermenting *S. cerevisiae* in the early 1900's.<sup>1</sup> Until recently, little attention has been paid to the influence of the substituents attached to the nitro aromatic ring undergoing reduction.

In 1989, Takeshita *et al.* established some information about the substituent effects on the reduction of nitroarenes **97**.<sup>3</sup> The presence of an electron-withdrawing group substituted *ortho*, *para* or *meta* to the nitro group on the aromatic ring resulted in the reduction proceeding smoothly to afford the amino derivatives **98** in yields ranging from 61-88 %. In contrast, the presence of an electron-donating group resulted in little or no reduction taking place with low yields (0-25 %) obtained (Fig 1-40).

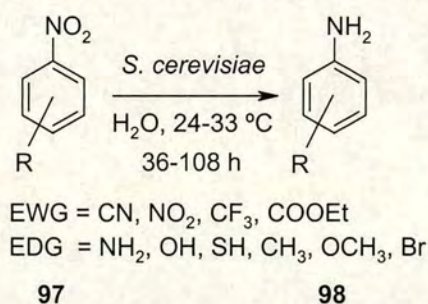


Fig 1-40 : *S. cerevisiae* catalysed reduction of substituted nitroarenes.

The same substituent effect was found to be present in the reduction of nitro groups substituted on heteroaromatics **99**, **100**, **101**.<sup>4</sup> An electron-donating group substituted



*ortho*, *para* or *meta* to the nitro group resulted in little or no reduction taking place, whereas the presence of an electron-withdrawing group greatly enhanced the rate of reduction of the nitro group with yields ranging from 41-95 % (Fig 1-41).

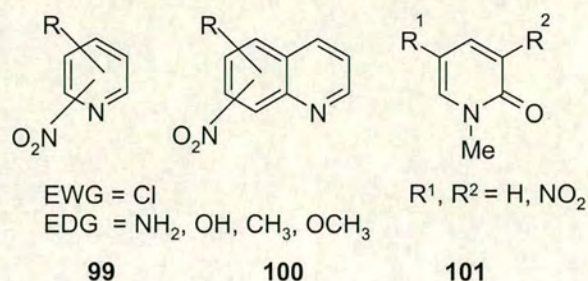


Fig 1-41 : *S. cerevisiae* catalysed reduction of nitro-heteroaromatics.

Davey *et al.* have reported on the regioselective reduction of substituted dinitroarenes **102** affording low yields of reduced products **103** (7-35 %) (Fig 1-42).<sup>2</sup>

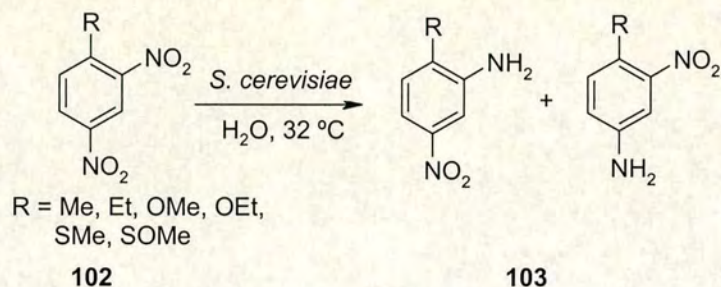


Fig 1-42 : *S. cerevisiae* catalysed reduction of dinitroarenes.

The regioselectivity was controlled by two competing factors, steric and electronic; the most sterically demanding R-substituted group directed reduction towards the *para* position, but the presence of a lone pair on the R-group directed reduction to the *ortho* position (Fig 1-43).

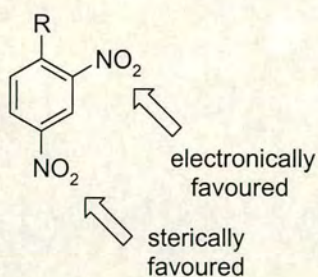


Fig 1-43 : Model representing the selectivity in dinitroarenes.



Baik *et al.* have reported on the selective and rapid reduction of aromatic nitroarenes **104** to afford **105** using *S. cerevisiae* under a slightly unusual set of reaction conditions: a basic solution with methanol or ethanol as a co-solvent and a high temperature was used (Fig 1-44).<sup>65</sup>

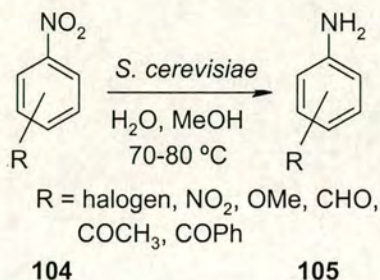


Fig 1-44 : *S. cerevisiae* catalysed reduction of nitroarenes under basic conditions.

High yields of the corresponding *ortho*, *para* and *meta* substituted anilines **105** were reported despite the presence of other reducible (carbonyl) and labile groups and only one nitro group was selectively reduced when *para*-dinitrobenzene was used as the substrate. Surprisingly, electron-withdrawing groups substituted on the aromatic ring **104** were tolerated, although the yields of the corresponding reduced products **105** were low (18-25 %).

It is known that the reduction of nitro compounds to the corresponding amine proceeds through intermediate stages involving nitroso and hydroxylamine compounds.<sup>66, 67</sup> Baik *et al.* have investigated the reductive cyclisation of *ortho*-nitrophenylazodyes **106** using *S. cerevisiae* under basic conditions to determine the intermediacy of the reaction pathways (Fig 1-45).<sup>68</sup>

The *N*-oxide intermediate **107** was isolated and underwent subsequent reduction to the benzotriazole **108** in 90 % yield when twice the amount of *S. cerevisiae* and longer reaction times were employed. This study revealed that this reduction pathway proceeded *via* an *N*-oxide intermediate **107** which went on to afford the triazole **108**. It was concluded that simple nitro aromatics underwent reduction so fast to the corresponding amine product that intermediate(s), e.g. nitroso species could not be detected.



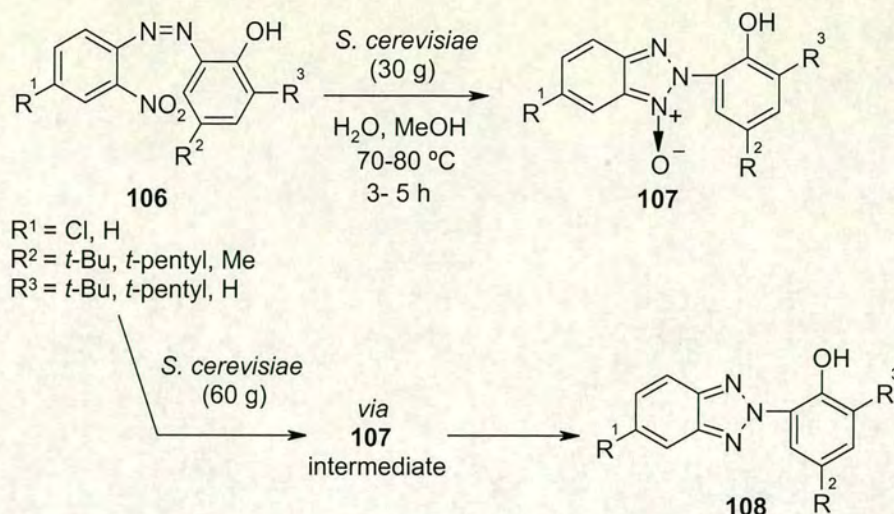


Fig 1-45 : *S. cerevisiae* catalysed reductive cyclisation under basic conditions.

Blackie *et al.* subsequently commented that two distinct sets of conditions were being employed for the reduction of nitroarenes mediated by *S. cerevisiae*.<sup>69</sup> Most of the literature described the use of type 1 conditions (Takeshita *et al.*) whereas Baik reported type 2 conditions (Table 1).

	Type 1 conditions	Type 2 conditions
<b>Temperature</b>	30 °C	70-80 °C
<b>pH</b>	5.5-6.0	>12 (high)
<b>Work-up</b>	None	Some cases, involving reflux
<b>Co-solvent</b>	None	Methanol/ethanol

Table 1 : Two distinct types of conditions for nitro reduction using *S. cerevisiae*.

These complementary reaction conditions led to different selectivity in the reduction of nitroarenes. Under the type 1 conditions, electron-donating groups could not be tolerated (low yields of product were isolated); electron-withdrawing groups were required for reduction to proceed smoothly and in high yield. In the latter set of conditions, electron-donating groups could be used, affording aniline derivatives.



The reduction of nitrobenzene **109** was used to discriminate between the two sets of reaction conditions (Fig 1-46, 1-47).

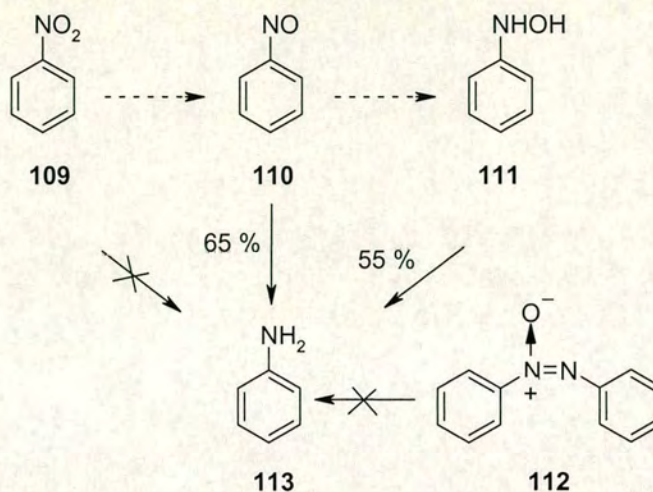


Fig 1-46 : *S. cerevisiae* catalysed reduction of nitrobenzene and derivatives using type 1 conditions.

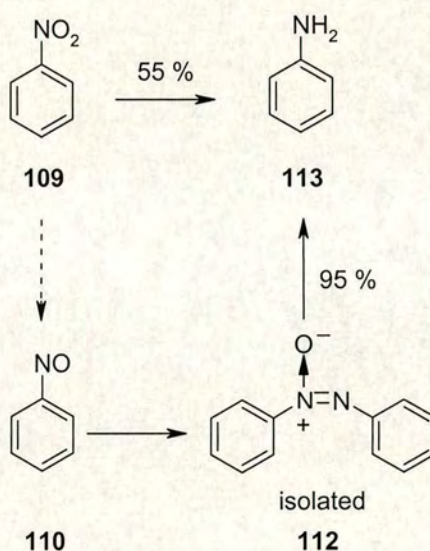


Fig 1-47 : *S. cerevisiae* catalysed reduction of nitrobenzene and derivatives using type 2 conditions.

Examination of both reaction mechanisms highlighted differences in the reduction of the same substrates (Table 2).



Substrates	Type 1 conditions	Type 2 conditions
Nitrobenzene <b>109</b>	No reduced product was detected	Aniline <b>113</b> was obtained
Nitrosobenzene <b>110</b>	Clean conversion to aniline <b>113</b>	Azoxybenzene <b>112</b> detected <i>en route</i> to aniline
Phenylhydroxylamine <b>111</b>	Aniline <b>113</b> was obtained	No indication of presence
Azoxybenzene <b>112</b>	No reduction	Aniline <b>113</b> was obtained

Table 2 : Differences observed in the reduction of simple nitroarenes.

Differences in reaction mechanisms when employing both types of conditions were evident from the investigation of the reduction of nitrobenzene **109** and other similar substrates **110**, **111**, **112**. For instance, azoxybenzene **112** was not reduced under type 1 conditions whereas it was detected *en route* to aniline **113** under type 2 conditions and thus proposed as an intermediate in the reduction pathway.

Further study of type 2 reaction conditions led to the conclusion that *S. cerevisiae* was simply acting as a source of carbohydrate and that the reduction was in fact non-enzymatic. It seemed very unlikely that the *S. cerevisiae* cells remained active at high temperatures and high pH, since it is known that simple nitroarenes can be reduced to the corresponding anilines and azoxybenzene compounds under strong basic conditions in solutions that contain alcohol and/or glucose/fructose.<sup>70-72</sup>

### 1.3.2 Nitroalkenes.

A method for the formation of saturated nitroalkanes could involve the reduction of  $\alpha$ - $\beta$ -unsaturated nitro aromatic compounds using *S. cerevisiae*. Takeshita *et al.* carried out studies on the chemoselective reduction of aromatic and heteroaromatic nitroalkenes using *S. cerevisiae*.<sup>5</sup>



The presence of an electron-withdrawing group (Br, Cl, CN) substituted on the ring **114** afforded the corresponding *ortho* and *meta* substituted saturated nitroalkanes **115** in yields of 41-81 % with the reduction proceeding smoothly (Fig 1-48).

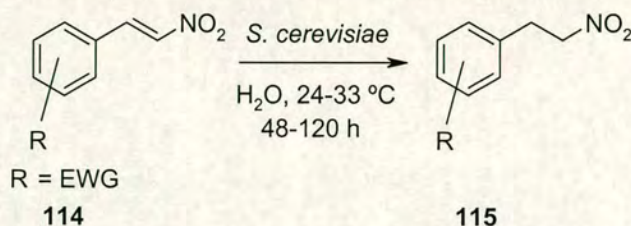


Fig 1-48 : *S. cerevisiae* catalysed reduction of substituted nitrostyrene.

Selective reduction of the nitro group substituted on the aromatic ring occurred in methyl substituted nitrostyrenes **116** to afford the corresponding aniline derivatives **117** as the major products (16-61 % yield) and the nitroalkanes **118** were obtained in low yields (10-24 % yields) (Fig 1-49).

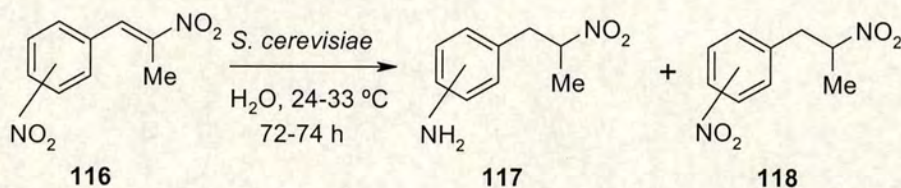


Fig 1-49 : *S. cerevisiae* catalysed reduction of methyl-substituted nitroalkenes.

Heteroaromatic and 5-membered ring systems **119** (thiophene, furan and pyrrole) were also studied. It was found that the carbon-carbon double bond was selectively reduced to afford the corresponding nitroalkanes **120** in moderate yields irrespective of what substituent was present on the heteroaromatic ring (Fig 1-50).

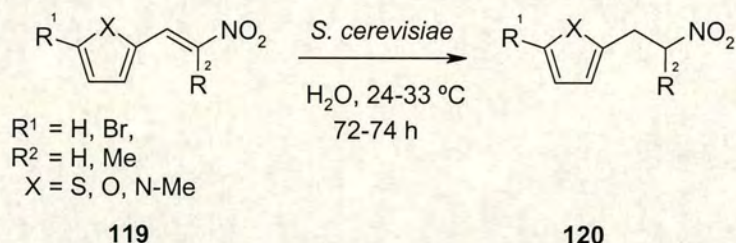


Fig 1-50 : *S. cerevisiae* catalysed reduction of heteroaromatic ring systems.



### 1.3.3 Isoxazoles.

An efficient synthesis of substituted isoxazoles **122** can be mediated by *S. cerevisiae* acting on nitroalkenes **121** with moderate yields in the range of 50-82 % obtained (Fig 1-51).<sup>6</sup>

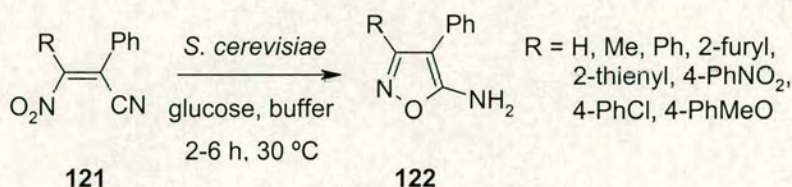


Fig 1-51 : *S. cerevisiae* catalysed reduction of substituted nitroalkenes.

The initial step was presumed to involve the reduction of the vinylic nitro group **121** to the hydroxylamine derivative which underwent cyclisation to form the 3-alkyl- and 3-aryl-4-aryl-5-amino-isoxazoles **122**.

The reductive cleavage of the N-O bond in substituted isoxazoles **123** has been reported affording the ring-opened compounds **124** in modest yields (Fig 1-52).<sup>73</sup> *S. cerevisiae* has been used as an alternative to metal catalysis in the cleavage of this N-O bond in cases when other groups susceptible to reduction were present.

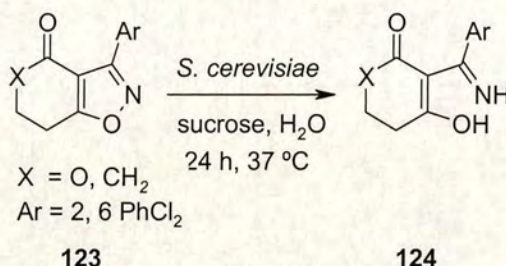


Fig 1-52 : *S. cerevisiae* catalysed reduction of substituted isoxazoles.

### 1.3.4 N-Oxides.

Another class of compounds that have been shown to undergo reductive cleavage of the N-O bond are *N*-oxides. In this case, substituted aromatic *N*-oxides **125** were reduced to the corresponding deoxygenated derivatives **126** (Fig 1-53).<sup>7</sup>



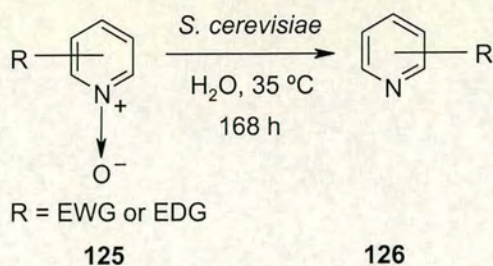


Fig 1-53 : *S. cerevisiae* catalysed reduction of pyridine *N*-oxides.

An electron-donating group (Me, OH, OMe) substituted on the pyridine ring **125** resulted in the reduction proceeding smoothly to afford the deoxygenated products **126** in yields of 22-44 %. In contrast, the reaction proceeded with poor yields (0-7 %) when an electron-withdrawing group (NO<sub>2</sub>, CN or Br) was substituted. Aliphatic *N*-oxides were reduced in very good yields (60-80 %) regardless of what groups were present.

Upon incubation of acetylpyridine *N*-oxide **127** with *S. cerevisiae*, the carbonyl group was reduced in preference to the N-O bond to afford the alcohol **128** with (*S*)-configuration (Fig 1-54).

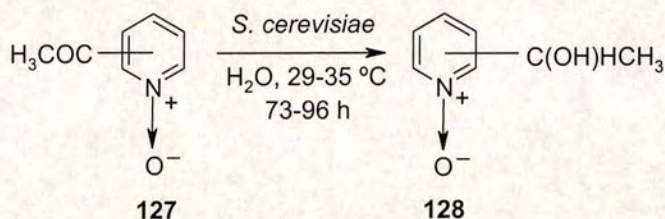


Fig 1-54 : *S. cerevisiae* catalysed asymmetric reduction of acetylpyridine *N*-oxides.



### 1.3.5 Conclusion.

The reductive cleavage of N-O bonds mediated by *S. cerevisiae* in substrates of many classes has been explored but further work is necessary to understand the reduction process employed by this whole-cell system.

In general, the biotransformations have been shown to proceed with high chemo-, regio- and stereo-selectivity under mild reduction conditions (Takeshita *et al.*).<sup>2-4, 6, 7</sup> The 'harsh conditions' that have been employed to effect 'difficult reductions' are postulated to result in a non-enzymatic process with the *S. cerevisiae* acting solely as a source of carbohydrate (Baik *et al.*).<sup>5, 65, 68, 74</sup>

## 1.4 *S. cerevisiae* mediated reduction of C=N and N=N containing groups.

### 1.4.1 Imines.

Imines are of particular interest as enzyme substrates because stereoselective reduction would afford either a (*R*)- or (*S*)-secondary amine depending on choice of the substrate having (*E*)- or (*Z*)-configuration.

Immobilised *S. cerevisiae* efficiently reduced the carbon-nitrogen double bond in *N*-benzylidinemethylamine **129** to afford the corresponding amine product **130** in the presence of aprotic solvents and 18-crown-6 (Fig 1-55).<sup>75</sup> The imine substrate **129** underwent decomposition in the presence of water and glucose with no reduction taking place.

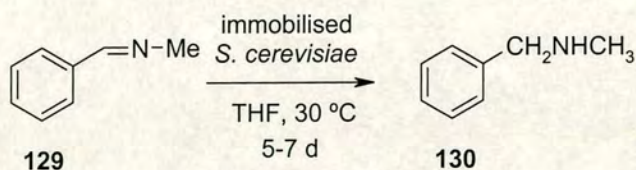


Fig 1-55 : *S. cerevisiae* catalysed reduction of imine derivative.



## 1.4.2 Azides.

*S. cerevisiae* can be employed for the chemoselective reduction of aryl azides **131** to aryl amines **132** with high yields (83-92 %) obtained under exceptionally mild reaction conditions (Fig 1-56).<sup>76</sup>

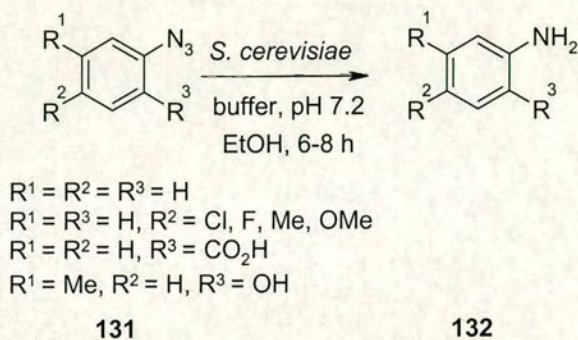


Fig 1-56 : *S. cerevisiae* catalysed reduction of aryl azides.

The biocatalytic reduction of azides has been extended to the synthesis of antitumour agents, the key step being the chemoselective reduction of 4-azidopodophyllotoxin **133** to **134** with *S. cerevisiae* which proceeded with excellent selectivity under mild conditions (Fig 1-57).<sup>77</sup>

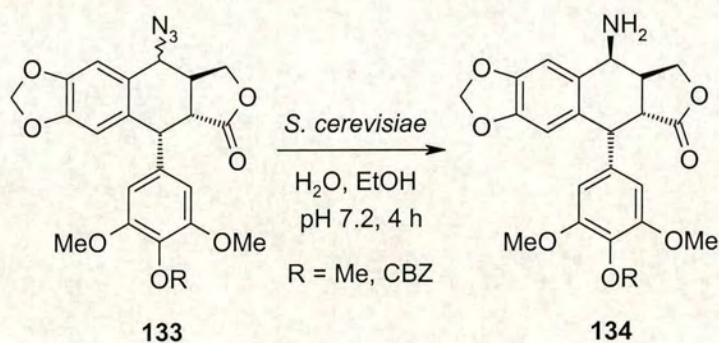


Fig 1-57 : *S. cerevisiae* catalysed chemoselective reduction of cyclic azides.

The reaction was high yielding (88 % and 84 % respectively) and chemoselective since the *ortho*-carboxybenzyl group (CBZ) remained totally intact to afford 4- $\beta$ -aminopodophyllotoxins **134**.



## 1.5 Chemical reduction of N-O containing groups.

Nitro groups can be reduced in two general ways:

- (a) Catalytic hydrogenation employing molecular hydrogen over a platinum or nickel catalyst and pressure maybe required.<sup>78</sup>
- (b) Acidic conditions employing heavy metals such as tin, zinc or iron.<sup>79</sup>  
Other reagents such as hydrazine (catalyst)<sup>80, 81</sup>, titanium trichloride<sup>82</sup> and sulfide derivatives<sup>83</sup> have also been reported.

The hydrogenation of a nitro compound **135** to the corresponding amine **136** takes place smoothly when a solution of the nitro compound is shaken with finely divided nickel or platinum under a hydrogen atmosphere (Fig 1-58).

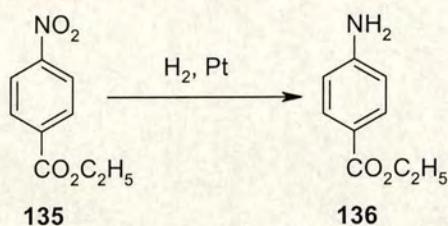


Fig 1-58 : Chemical reduction using hydrogen over a catalyst.

Molecular hydrogen cannot be employed chemoselectively when the molecule **137** contains other reducible groups such as a carbon-carbon double bond. Both the double bond and nitro group would be reduced to the saturated amine **138** product (Fig 1-59).

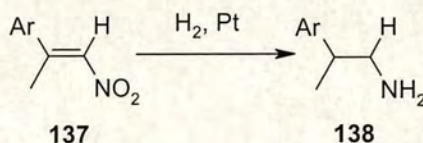


Fig 1-59 : Chemical reduction using hydrogen over a catalyst.

Chemical reduction in the laboratory is most often carried out by addition of an acid to a mixture of the nitro compound **139** and heavy metal. In the acidic solution, the amine **140** is obtained as its salt with the free amine **141** liberated by the addition of a base (Fig 1-60).



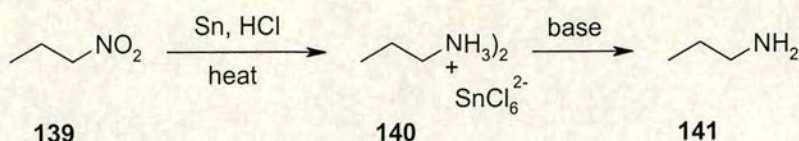


Fig 1-60 : Chemical reduction using heavy metal and acid catalysis.

A couple of reported examples which involve the reductive cleavage of the N-O bond are discussed below.

The synthesis of optically active alcohols with excellent stereoselectivity can be achieved by the reductive cleavage of the N-O bond in substituted hydroxylamines **142** to afford the (*R*)-allylic diols **143** in yields of 51 % and 94 % respectively with e.e. of 95 % (Fig 1-61).<sup>84</sup>

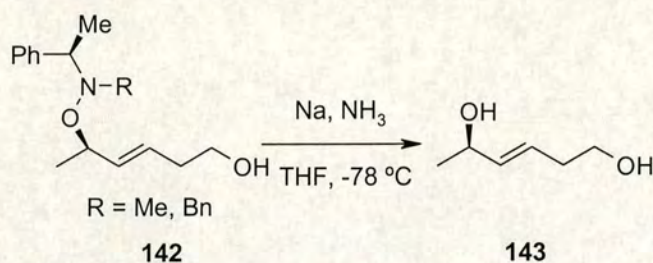
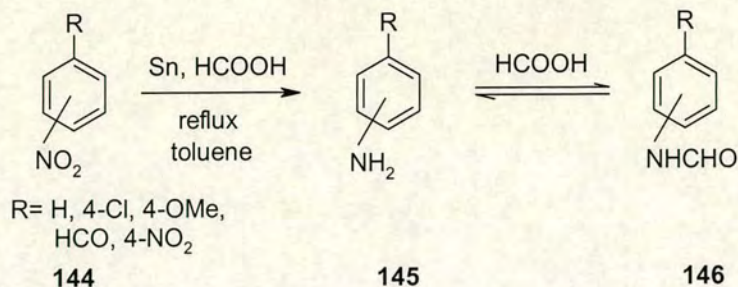


Fig 1-61 : Reductive cleavage of N-O bond in hydroxylamines.

A range of reducing reagents were employed without success which included hydrogenolytic procedures and finally sodium in liquid ammonia was found to afford the desired bond cleavage in a high chemical yield with excellent stereoselectivity.

Another paper has reported on the reduction of aryl nitro compounds **144** under acidic conditions to afford the corresponding amines **145** which are obtained as *N*-formamide compounds **146** in yields ranging from 47-86 % (Fig 1-62).<sup>85</sup> Both nitro groups of dinitrobenzene were reduced to form the diformamide in 86 % yield.





*Fig 1-62 :* Reductive cleavage of N-O bond in aryl nitro compounds.

### 1.6 Enzymatic reduction of NO<sub>2</sub> groups using other microorganisms.<sup>86</sup>

Nitro aromatic compounds are abundant in nature and are of considerable industrial importance in the production of plastics, pharmaceuticals, dyes and agricultural chemicals. Nitro compounds in most cases are highly toxic to the living organisms, so several microorganisms are capable of mineralising or converting nitro-aromatics. Natural nitro compounds are readily biodegradable and serve as sources of carbon, nitrogen and energy for the microorganisms. Several pathways and enzymes used by these microorganisms in the degradation and conversion of nitro aromatics are discussed below.

### 1.6.1 Monooxygenase : catalysed initial reduction.<sup>87</sup>

- Oxidative removal of the NO<sub>2</sub> group from the aromatic nucleus by the addition of single oxygen leading to the elimination of the NO<sub>2</sub> group in the form of a nitrite molecule.

A monooxygenase from *Pseudomonas putida* isolated from soil is responsible for the reduction of 2-nitro-phenol **147** to afford catechol **148** with the release of a nitrite molecule. The catechol **148** produced in this reaction could be further metabolised (Fig 1-63).



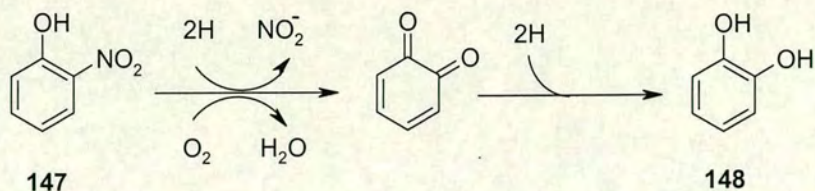


Fig 1-63 : Postulated pathway of 2-nitro-phenol degradation.

### 1.6.2 Dioxygenase : catalysed initial reduction.<sup>86</sup>

- Insertion of two hydroxyl groups into the aromatic ring with the elimination of the  $NO_2$  group.

A *Nocardia* sp. has been reported to mineralise 3-nitro-benzoate **149**, the initial step being the dioxygenase catalysed attack at the 3 and 4 position of the molecule with subsequent release of nitrite to form protocatechuate **150** (Fig 1-64).

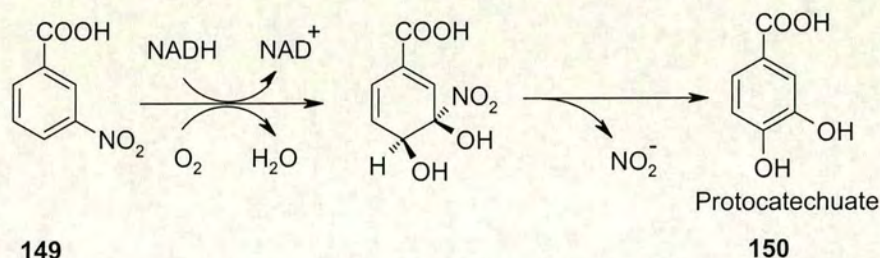


Fig 1-64 : *Nocardia* sp. catalysed degradation of 3-nitro-benzoate.

### 1.6.3 Reduction of the aromatic ring.<sup>86, 88</sup>

- Complete reductive elimination of the  $NO_2$  group in the form of a nitrite molecule.

The reduction of picric acid **151** by *Rhodococcus erythropolis* is believed to be initiated by the hydride attack on the aromatic ring, followed by decomposition to liberate the nitrite and generate 2,4-dinitro-phenol **152** (Fig 1-65).



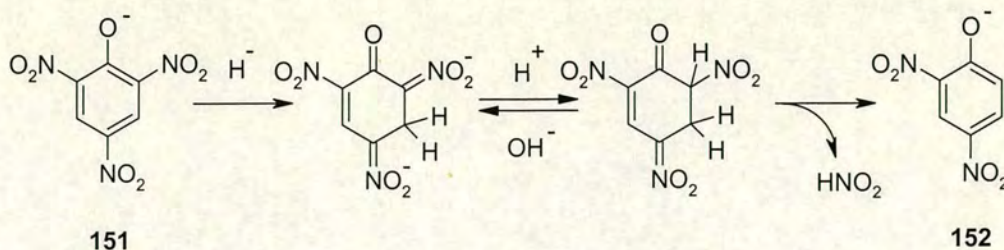


Fig 1-65 : Reduction of the aromatic ring in picric acid.

#### 1.6.4 Partial reduction of the $NO_2$ group.<sup>89, 90</sup>

- Formation of hydroxylamine (intermediate) which after subsequent replacement is further metabolised with the release of nitrogen from the molecule as ammonia.

The degradation of 4-nitro-benzoate **153** catalysed by *Comamonas acidivorans* NBA-10 under aerobic conditions showed an incomplete reduction of the nitro group (Fig 1-66). The hydroxylamine **154** was identified as an intermediate but was not reduced further to the expected 4-amino-benzoate product. Instead, hydroxyaminobenzoate **154** was converted to the dihydroxybenzoate **155** with the release of ammonia.

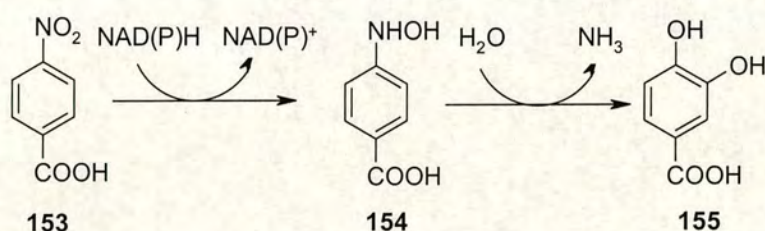


Fig 1-66 : Proposed pathway for the partial degradation of 4-nitro-benzoate.

The degradation pathway employed by this microorganism did not follow the general pattern of aerobic metabolism since no molecular oxygen was employed. Instead the hydroxyl groups of dihydroxybenzoate **155** were a result of water addition which led to the release of nitrogen from the molecule as ammonia.



### 1.6.5 Reduction to form amine.<sup>91</sup>

- The amine produced could be further metabolised.

A recent paper has reported on the complete reduction of the NO<sub>2</sub> group on a range of nitroarenes **147**, **153**, **156** to the corresponding amino-compounds by the use of a bacterial sludge bed reactor under anaerobic conditions (Fig 1-67). The amino compounds obtained were further mineralised to afford methane and carbon dioxide gas.

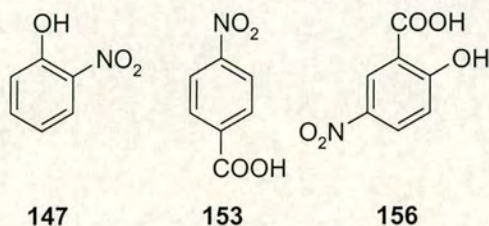


Fig 1-67 : Complete mineralisation of selected nitro-aromatics.

### 1.6.6 Miscellaneous (Nitro-reductases).

Some bacteria and fungi are able to reduce the NO<sub>2</sub> group of nitro-aromatics but cannot mineralise the compounds any further. Nitro-reductases from these bacteria and fungi have a wide spectrum of substrates in contrast to those reductases from microorganisms which are capable of utilising nitro-aromatics as the sole carbon and energy source.

A nitro-reductase purified from *Pseudomonas pseudoalcaligenes* JS45 catalysed the reduction of nitrobenzene **109** to hydroxy-amino-benzene **111** and not to the expected amino-benzene product (Fig 1-68).<sup>92</sup>

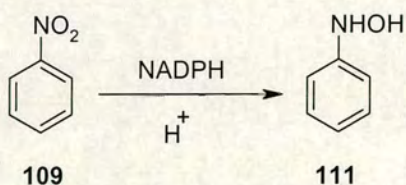


Fig 1-68 : Reduction catalysed by nitro-reductase.



The phototrophic bacterium *Rhodobacter capsulatus* E1F1 reduced 2,4-dinitrophenol **152** to 2-amino-4-nitrophenol **157** by a nitrophenol reductase activity which was present in this bacterium (Fig 1-69).<sup>93</sup> The reductase require NAD(P)H as an electron donor for the catalysed reduction of several nitro-phenol derivatives.

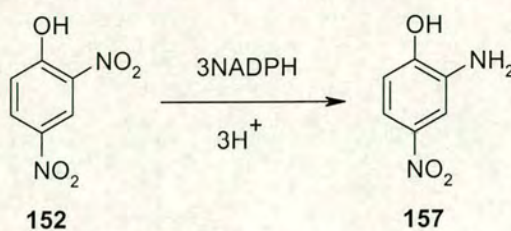


Fig 1-69 : Reduction catalysed by nitro-phenol reductase.

### 1.6.7 Nitro-reductases.

To our knowledge, very little has been reported on the reducing ability of enzymes isolated from *S. cerevisiae*. In our laboratories, 3 enzymes from *S. cerevisiae* have been isolated and purified. Each has been shown to reduce the nitro group of simple nitro aromatic compounds to the corresponding amino moiety.<sup>94</sup> This work will be discussed in Chapter 3. However, nitro-reductases isolated from other microorganisms afford the corresponding amino derivatives and have been discussed in section 1.6.6 and in Chapter 3.

### 1.7 Isolated enzymes.<sup>95</sup>

Advances in biocatalysis have recently been focused on the growing number of applications of enzymes in organic synthesis. This is a consequence of the increased availability of new biocatalysts for both laboratory and industrial scale use. The stereoselectivity exerted by a whole cell system on an artificial substrate is not always satisfactory: poor stereoselectivity is often the result of simultaneous action of several enzymes that exert opposite stereochemistry towards the substrate. In such a case, the use of an isolated enzyme is recommended in order to obtain a single enantiomer.



### 1.7.1 Reductases.

Recently, several enzymes have been isolated and characterised from *S. cerevisiae* and reported to have reducing capabilities which are discussed below.

The isolation of a Yeast Keto Ester Reductase-1 (YKER-1) from *S. cerevisiae* afforded the corresponding (2*R*, 3*S*, 1'*R*)-hydroxy esters **159** from **158** in excellent stereoselectivity (Fig 1-70).<sup>96, 97</sup> The keto ester reductase discriminated three chiral centres simultaneously producing useful chiral building blocks with e.e. and d.e of 98 % and 87 % respectively.

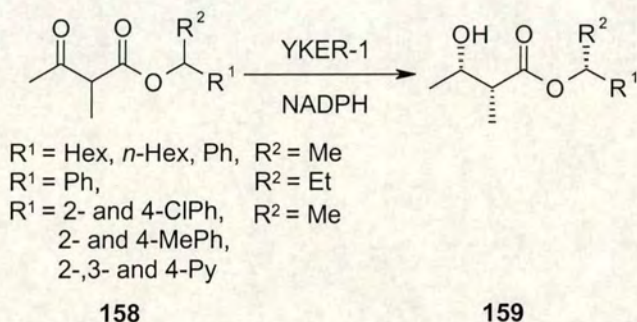


Fig 1-70 : YKER-1 catalysed reduction of  $\beta$ -keto esters.

YKER-1 is a very useful enzyme in the field of organic synthesis because of its excellent stereoselectivity and easy availability. The reductase can catalyse the formation of optically active hydroxy esters and hydroxy ketones from the corresponding keto esters and diketones (Fig 1-71).<sup>98</sup>

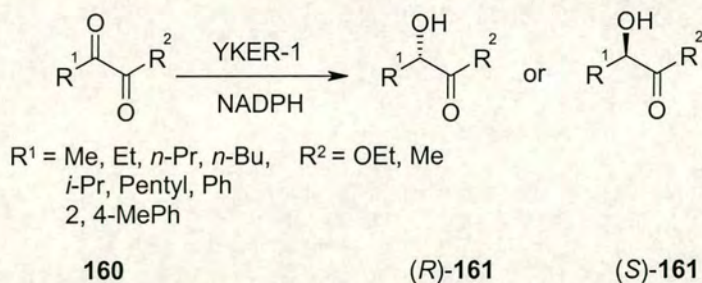


Fig 1-71 : YKER-1 catalysed reduction of  $\alpha$ -keto ester and  $\alpha$ -diketones.

The reduction of  $\alpha$ -keto esters **160** afforded the corresponding (*R*)-**161** or (*S*)-**161** hydroxy esters selectively with e.e. of 82 %. The stereochemical course depended on



the chain length of the alkyl substituent, a short chain ( $R^1$ ) afforded the (*S*)-configured hydroxy ester whereas a long alkanoate chain yielded the corresponding (*R*)-hydroxy ester. In contrast, the reduction of  $\alpha$ -diketones **160** only afforded the corresponding (*S*)-hydroxy ketone **161** with e.e. greater than 95 % irrespective of the chain length of the alkyl substituent.

An NADPH dependent reductase from *S. cerevisiae* displayed reducing activity towards several non-natural substrates **162** with excellent stereoselectivity, 98 % e.e. (Fig 1-72).<sup>99</sup>

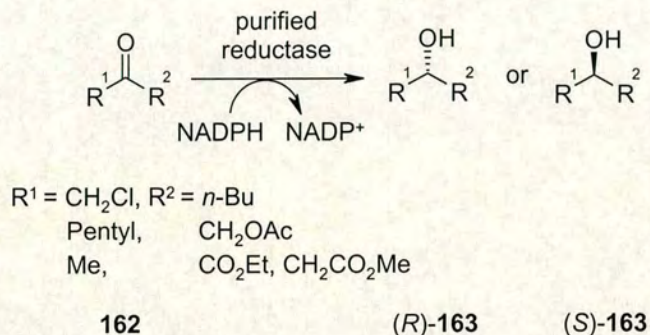


Fig 1-72 : Purified reductase catalysed reduction of ketones.

Substrates which contained chlorine ( $R^1$ ) afforded the corresponding optically active alcohols with (*R*)-configuration **163**, whereas the non-chlorinated substrates afforded the (*S*)-configured alcohol products **163**.



## 1.8 Nitrilases mediated biotransformations.<sup>100-103</sup>

Organic compounds containing the cyano group (CN) are synthesised in plants, animals, insects and fungi and hence are numerous and widespread throughout the environment. Nitriles are also products of petrochemical solvents, recrystallising agents and chiral synthons. The highly reactive CN functional group is toxic to living cells in nature and is able to inhibit biochemical pathways. The compounds are highly significant intermediates in organic synthesis for the preparation of amines, amides and carboxylic acids/esters (Fig 1-73).

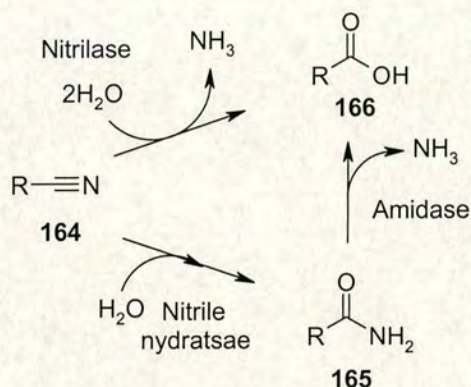


Fig 1-73 : Nitrile hydrolysis using hydratases and nitrilases.

The most common reaction for the microbial metabolism of the nitrile compound is the hydrolysis of the CN group. It is recognised that there are two enzymatic pathways available (Fig 1-73). Aliphatic nitriles **164** are generally metabolised in two stages: Firstly, they are converted to the corresponding amide **165** by a nitrilase hydratase and then an amidase converts the amide **165** to the corresponding carboxylic acid **166** and ammonia.

Aromatic, heterocyclic and certain unsaturated aliphatic nitriles **164** are hydrolysed directly without the formation of the amide intermediate to the acid **166** with the release of ammonia which is catalysed by a nitrilase. The enzymology of microbial nitrile metabolism is well understood and the main enzymes that take part in the metabolism are discussed below.



### 1.8.1 Nitrilase hydratases.<sup>100, 103</sup>

Of all the nitrilase hydratase activities reported so far, the enzymes in the genus *Rhodococcus* have been characterised the most, both with respect to their biochemical properties and their growth conditions required for optimal expression. Nitrile hydratases have been described in many bacteria including *Rhodococcus* spp.<sup>104, 105</sup>, *Pseudomonas*<sup>106</sup>, *Agrobacterium*<sup>107, 108</sup> and *Corynebacterium* spp.<sup>109</sup>

Nitrilase hydratases are known as metalloenzymes, which possess a tightly bound divalent metal ion, either cobalt or iron that is required for catalysis. The reaction sequence for the conversion of a nitrile **164** to the amide **165** is catalysed by this class of enzymes.

As interest in the biotechnological applications of nitrile transforming enzymes grew, it was inevitable that those enzymes capable of the partial hydrolysis of acrylonitrile **167** to the important commercial product acrylamide **168** would be among the first sought (Fig 1-74).<sup>110, 111</sup>

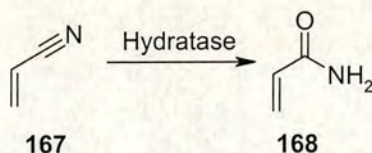


Fig 1-74 : Nitrile hydratase catalysed hydrolysis of acrylonitrile.

The Nitto Chemical Industry in Japan, started the industrial production of acrylamide **168** through a biotechnological route in 1985 using *Corynebacterium* sp. N-774. The productivity of this Nitto process was further enhanced in 1991 by another powerful biocatalyst, *Rhodococcus rhodochrous* J1. This microorganism, which contained a cobalt nitrile hydratase, was superior in terms of activity and productivity with the recovery of unreacted acrylonitrile unnecessary as the conversion was more than 99.99 %.



1.8.2 Nitrilases.<sup>103</sup>

Discovered 40 years ago, the distribution of the nitrilases appear to be wider than that of the nitrilase hydratases but they have been studied in less detail. Early work concentrated on the nitrilases which catalysed the production of acrylamide whereas nitrilases convert nitriles directly into the carboxylic acid with the release of ammonia and no accumulation of the amide. Nitrilases have been described in many bacteria including *Pseudomonas*<sup>112, 113</sup>, *Nocardia* spp.<sup>114, 115</sup> and *Rhodococcus* spp.<sup>116</sup>

Nitrilases employ a different mechanism compared to the nitrilase hydratases, where the co-ordination of metals or co-factors is not required. Instead important cysteine residues within the enzyme take part in the hydrolysis process (Fig 1-75).

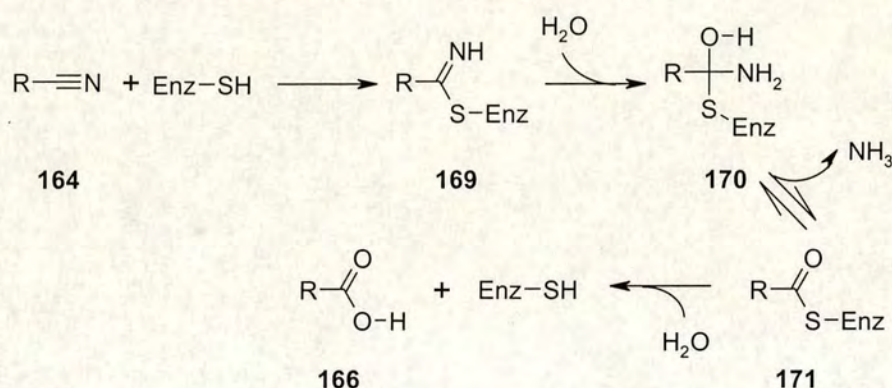


Fig 1-75 : Proposed mechanism of nitrilase catalysed nitrile hydrolysis.

Initially, a nucleophilic attack of the thiol from the cysteine residue within the enzyme onto the carbon of the nitrile molecule **164** affords an enzyme-bound imine **169**. This is hydrolysed to yield a tetrahedral intermediate **170** which loses ammonia to form an acyl-enzyme complex **171**, finally hydrolysis occurs liberating the carboxylic acid **166**.



### 1.8.3 Amidases.<sup>103</sup>

Amidases are a subgroup of proteases which hydrolyse carboxamides to yield the corresponding acids. A number of amidases actively participating in the hydrolysis of amides have been found to have diverse substrate specificity and are always linked with nitrile hydratases. Amidases have been described in many bacteria including *Arthrobacterium*<sup>80</sup>, *Rhodococcus* spp.<sup>117</sup> and *Brevibacterium* spp.<sup>118, 119</sup>

Interestingly, besides hydrolysing amides **165** different amidases have been reported to exhibit acyl transfer activity in the presence of hydroxylamine. This has been described for the amidases of *Rhodococcus* sp. R312 which afforded a range of hydroxamic acids **172** (Fig 1-76).<sup>120</sup>

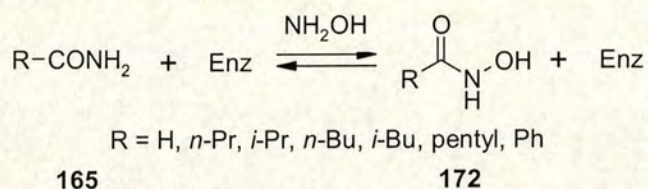


Fig 1-76 : Amidase catalysed acyl transfer on hydroxylamine.

### 1.8.4 Hydrolysis of nitriles.

Recently, there has been considerable interest in carrying out biotransformations in low water systems. The main advantage of this is that the concentration of poorly water-soluble substrates can be greatly increased, however problems arise from the fact that a number of enzymes are denatured in the presence of organic solvents. Enzymatic hydrolysis in low water systems using a nitrilase hydratase from *Rhodococcus* sp. DSM 11397 and a nitrilase from *Pseudomonas* DSM 11387 has been studied by Layh and Willetts.<sup>121</sup>

Both these bacteria retained their activity in organic/aqueous biphasic mixtures with hexadecane solvent (50-100 %, v/v) giving the best activity. Less hydrophobic solvents caused a significant loss of activity in both bacteria. In addition,



*Rhodococcus* sp. retained some activity in water-saturated (buffer) organic monophasic systems with the best results obtained using hexanol or decanol as the organic solvent.

Graham *et al.* have reported on the performance of free and immobilised cells of a *Bacillus* spp. which contained both a nitrile hydratase and an amidase and was capable of transforming a variety of straight and branched aliphatic and cyclic nitriles **173** to the corresponding acids (Fig 1-77).<sup>122, 123</sup>

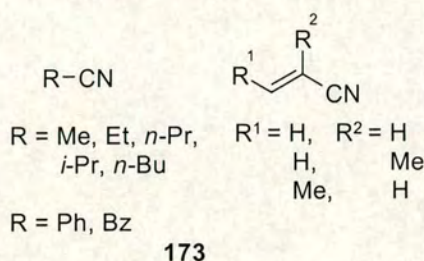


Fig 1-77 : *Bacillus* spp. catalysed hydrolysis of mono-nitriles.

In addition, the *Bacillus* bacterium could be employed in amide synthesis when used in combination with urea, which inhibited the amidase activity of the bacterium.

A recent paper has reported on the nitrilase activity of an amidase from *Rhodococcus rhodochrous* J1. The amidase catalysed the hydrolytic cleavage of the carbon-nitrogen triple bond in benzonitrile (R = Ph) **164b** to afford the corresponding acid **166** with the release of ammonia.<sup>124</sup> Interestingly, no corresponding acid product **166** was detected when benzamide (R = Ph) **165** was incubated with the amidase.

Considering the acyl-enzyme mechanism of amidase and its nitrilase activity, a mechanism to explain the above results (which was likely to be analogous to that of the nitrilase) was proposed (Fig 1-78).



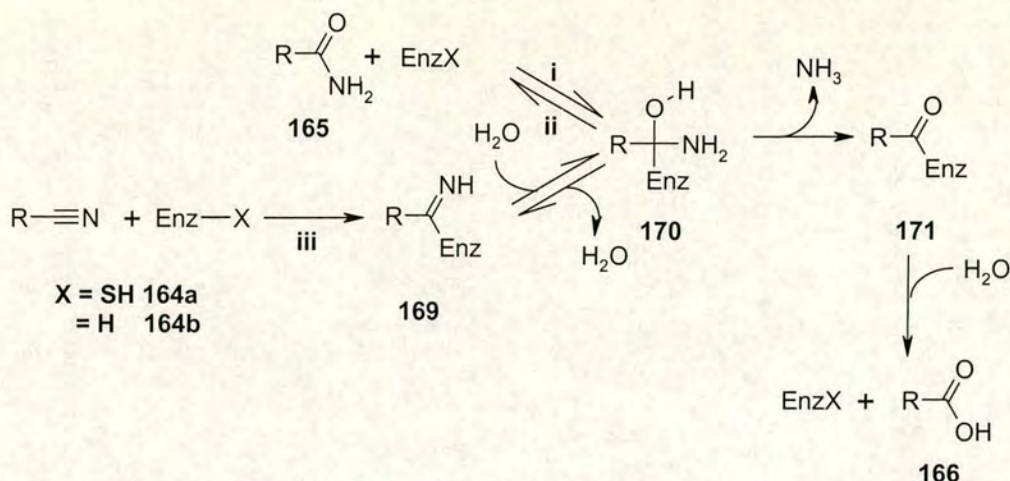


Fig 1-78 : Proposed mechanism of nitrilase/amidase catalysed hydrolysis.

When an amide **165** was used as the substrate in an amidase reaction, the carbonyl group of the amide would undergo attack by the nucleophilic part of the amidase enzyme (**route i**) resulting in the formation of a tetrahedral intermediate **170**. The release of ammonia would result in the formation of an acyl-enzyme bound molecule **171** which could be hydrolysed to the corresponding acid **166**.

No nitrilase activity was detected for the amidase, therefore when a nitrile **164b** was used as a substrate, the same nucleophilic part of the amidase enzyme (with amide as a substrate) would be nucleophilic for the carbon-nitrogen triple bond of the nitrile substrate (**route iii**) to form the acid product **166**.

The same authors have also reported on the hydrolytic activity of a nitrilase to amides present in *Rhodococcus rhodochrous* J1.<sup>125</sup> The nitrilase was known to catalyse the hydrolytic cleavage of the carbon-nitrogen triple bond in nitriles **164a** to afford the corresponding acids **166**. Surprisingly, however it was also found to catalyse the hydrolysis of specific amide substrates (benzamide) **165** to the corresponding acids **166**.

In the nitrilase catalysed reaction (Fig 1-78), the nucleophilic attack on the nitrile carbon **164a** by the nitrilase enzyme leads to the formation of a tetrahedral intermediate **170** via an enzyme-bound imine **169** (**route iii**). The tetrahedral



intermediate **170** could be broken down to produce an amide **165** (route ii) instead of the expected acid product **166**. The authors proposed that routes (i) and (ii) took place with nitrilases only under specific reaction conditions and with specific substrates to afford the amide product **165**.

*Rhodococcus rhodochrous* IFO 15564 contains both a nitrile hydratase and an amidase. The amidase activity can be inhibited by a specific functional group present on a molecule.<sup>126</sup> The boric acid **174** was hydrolysed to the amide **175** in 75 % yield (Fig 1-79i). Subsequent palladium cross coupling afforded the dienes **176** and re-treatment with the same microorganism afforded the corresponding acid **177** (87 %) (Fig 1-79ii).

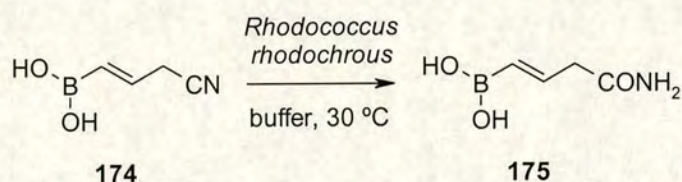


Fig 1-79i : *Rhodococcus rhodochrous* IFO 15564 catalysed nitrile hydrolysis.

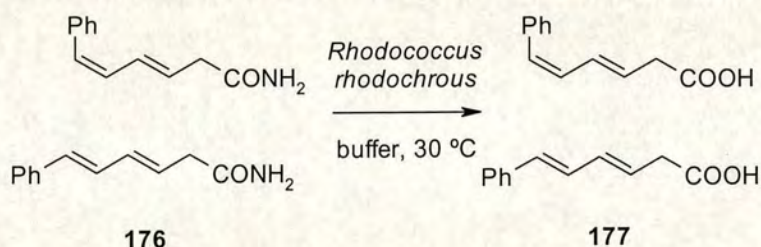


Fig 1-79ii: *Rhodococcus rhodochrous* IFO 15564 catalysed amide hydrolysis.

It was proposed that the inactivity of the amidase enzyme in the first transformation (Fig 1-79i) was due to the presence of the boronic acid moiety on the substrate **174** otherwise the corresponding acid product would have been obtained.

A versatile building block for the synthesis of new antituberculous agents has been prepared by whole-cell biotransformations. 2-Cyanopyrazine **178** was hydrolysed to the pyrazine carboxylic acid **179** using a nitrilase present in *Agrobacterium* sp. DSM



6336 and then hydroxylated using a dehydrogenase to afford 5-hydroxy-pyrazine-2-carboxylic acid **180** (Fig 1-80).<sup>127</sup>

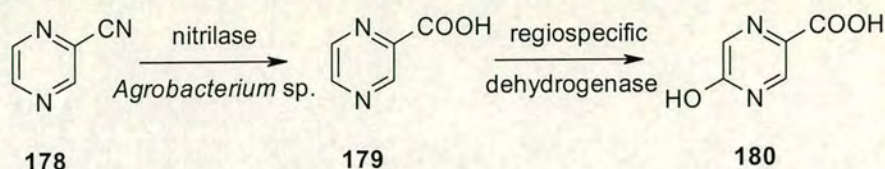


Fig 1-80 : *Agrobacterium* sp. catalysed hydrolysis of 2-cyanopyrazine.

Meth-Cohn and Wang have reported an in depth study on the hydrolysis of a variety of aliphatic **181**, di-substituted **182** and tri-substituted **183** aromatic nitriles into amides and/or acids using *Rhodococcus* sp. AJ270 under mild reaction conditions (Fig 1-81).<sup>128</sup>

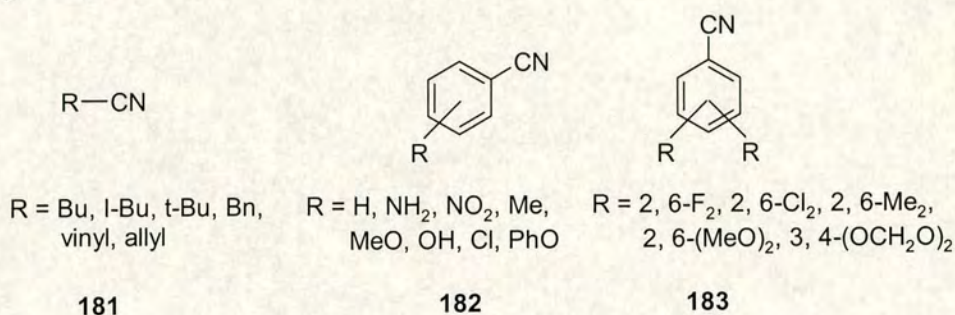


Fig 1-81 : *Rhodococcus* sp. AJ270 catalysed nitrile hydrolysis.

*Para*- and *meta*- substituted mono-benzonitriles **182** were hydrolysed efficiently to afford the corresponding acids (irrespective of the nature of the substituents attached) with yields ranging from 28-99 %. The amide product was produced when the more sterically hindered compounds **183** were employed. Saturated and unsaturated aliphatic nitriles **181** were hydrolysed smoothly to the corresponding acids in yields ranging between 60-99 %.

### 1.8.5 Enantioselective hydrolysis of nitriles.

The enantioselectivity of hydrolysing reactions carried out by nitrilases have been investigated only recently with both nitrilases and nitrile hydratases found to be relatively unspecific with respect to the chirality of the substrate. Generally, no



stereoselectivity was identified in the nitrilase biotransformation route, both with regard to the initial hydrolysis and the direct hydrolysis of the nitrile.

However, amidases have been known to produce enantiomerically enriched forms of products from the kinetic resolution of racemic substrates and play an important role in amino-acid synthesis.<sup>18</sup> As a rule the 'natural' (*L*)-configured enantiomer from the racemic ester **184** was usually converted to the acid **185**, leaving the (*D*)-enantiomer **186** behind (Fig 1-82).

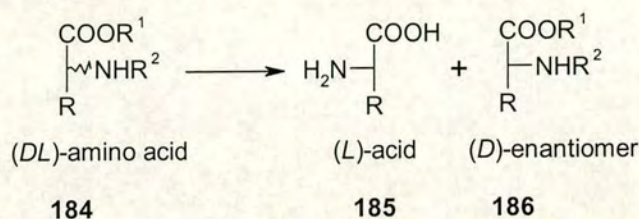


Fig 1-82 : Amidase catalysed hydrolysis of an amino acid.

Wang *et al.* have reported on the use of *Rhodococcus* sp. AJ270 as an efficient biocatalytic system which is capable of stereoselective conversion of racemic  $\alpha$ -substituted phenylacetonitriles **187** and amides **188** to the enantiopure carboxylic acids and derivatives under mild reaction conditions (Fig 1-83).<sup>129</sup>

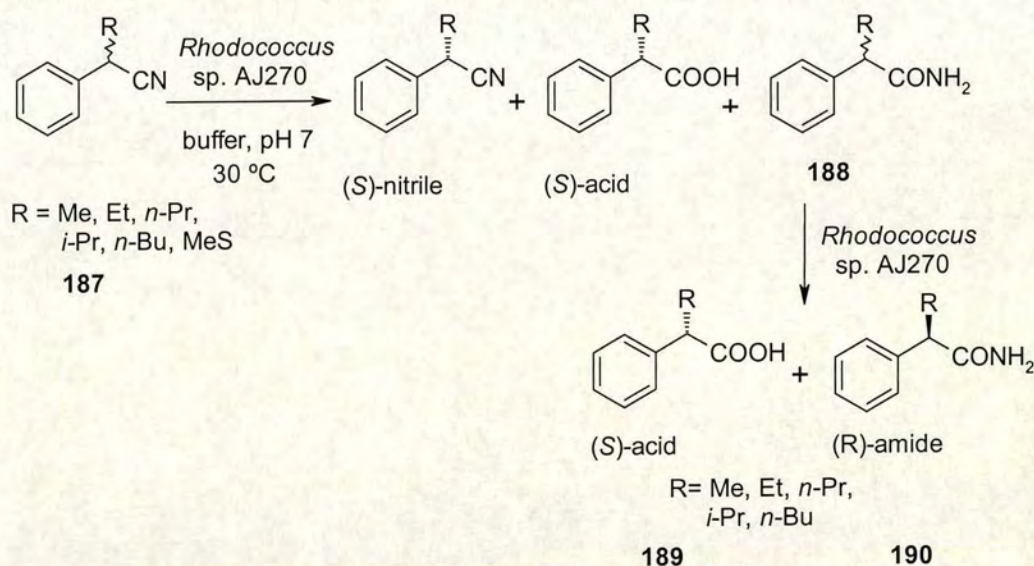


Fig 1-83 : *Rhodococcus* sp. AJ270 catalysed enantioselective hydrolysis.



Whilst the nitrile hydratase present in this bacterium displayed low (*R*)-selectivity when aliphatic nitriles were used as substrates, it was found to have a broad substrate spectrum with phenylacetoneitriles irrespective of the electronic nature of the  $\alpha$ -substituent. The amidase was sensitive to both the electronic and steric factors of the  $\alpha$ -substituted amides **188** for which it displayed high (*S*)-enantioselectivity **189** against amides. Unfortunately the opposite selectivity of the nitrile hydratase and amidase led to very low e.e.'s of (*R*)-amides **190** (3-9 %) obtained.

Optically active  $\alpha$ -substituted carboxylic acids (amino acids and hydroxy-carboxylic acids) have various biological functions and usually only one enantiomer is metabolically effective. Stolz *et al.* have reported on the attempts to obtain new nitrile converting enzyme systems from nature which possess the ability to convert racemic nitriles or amides to the corresponding optically active carboxylic acids.<sup>130</sup>

Of all the enzymes screened, the purified amidase from *Rhodococcus erythropolis* MP50 hydrolysed 2-phenylpropionamide **191a**, ketoprofen amide **192a** and naproxen **193** with excellent e.e. of 99 % and up to 49 % conversion of the respective substrates (Fig 1-84).

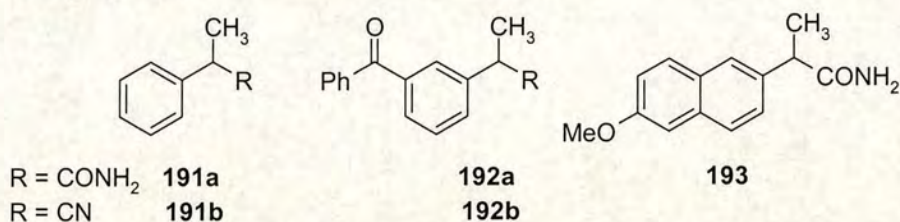


Fig 1-84 : Catalysed hydrolysis of selected nitriles and amides.

In addition, the partially purified nitrile hydratase from *Agrobacterium tumefaciens* d3 hydrolysed 2-phenylpropionitrile **191b** and ketoprofen nitrile **192b** to the corresponding (*S*)-amides with e.e. of 90 % and up to 30 % conversion of the respective substrate.

*Rhodococcus rhodochrous* IFO 15564 mediated hydrolysis of a range of cyanocyclohexane derivatives has been studied.<sup>131</sup> The nature and polarity of the substituents affected the activity of the amidase but not the nitrile hydratases that



were present in the microorganism. The typical cyclic-nitrile **194** gave the (*R, R*)-amide **195** in 41 % yield, with e.e. of 99 % and the (*S, S*)-acid **196** in 49 % yield, with e.e. of 73 % (Fig 1-85).

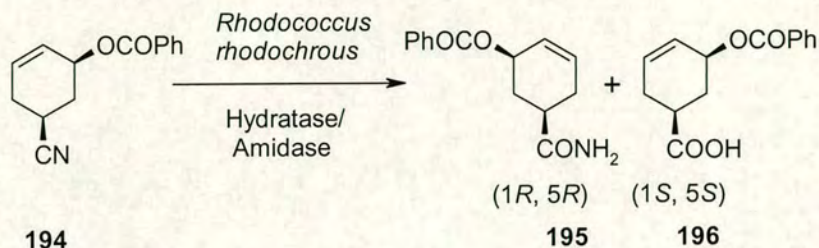


Fig 1-85 : *Rhodococcus rhodochrous* IFO 15564 catalysed hydrolysis of cyclic nitriles.

### 1.8.6 Regioselective hydrolysis of dinitriles.

The selective hydrolysis of one nitrile group out of several in a molecule is generally very difficult to do using traditional chemical catalysis, often resulting in the formation of complex product mixtures. In contrast, the use of microbial whole-cells can be very efficient for this transformation.

The hydrolysis of dinitriles **197** by nitrile metabolising enzymes presents an interesting means of synthesising a wide range of organic compounds not amenable to preparation in high yields by the conventional chemical methods. In addition, 5 different potential types of product **198**, **199**, **200**, **201** and **202** could be afforded in high yields when these enzymes are used, whereas only 2 products, a diamide **198** and diacid **199**, are possible with chemical synthesis (Fig 1-86).<sup>102</sup>



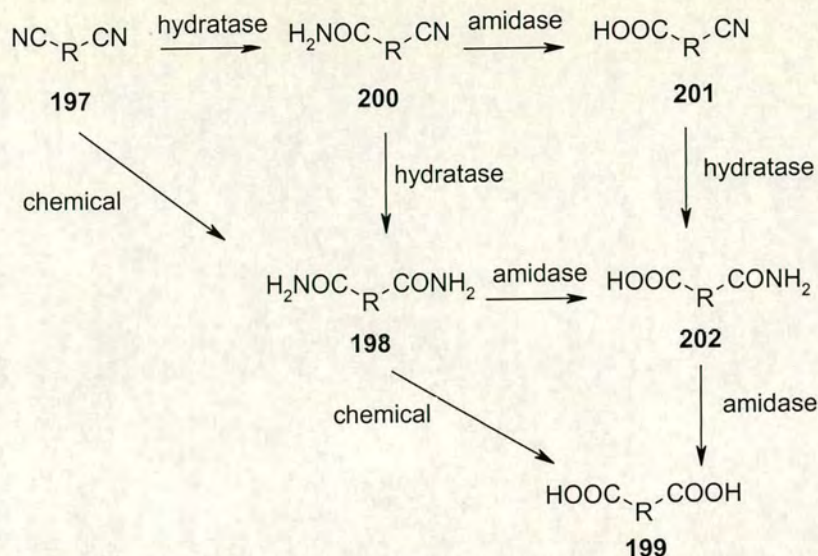


Fig 1-86 : Combined hydrolysis of dinitriles using nitrilases.

Microbial cells displaying aliphatic nitrilase activity (*Acidovorax facilis*) or a combination of nitrile hydratase and an amidase activity (*Comamonas testosteroni*) have been employed in the preparation of 5- and 6-membered ring lactams.<sup>132</sup> The enzyme-catalysed hydrolysis of aliphatic  $\alpha$ - and  $\omega$ -dinitriles **203** occurred at the primary cyano group to afford the corresponding cyano acids as their ammonium salts **204** in high yields (83-100 %). The ammonium salt **204** was converted directly to the corresponding *N*-alkyl-lactam **205** by hydrogenation in aqueous solution (Fig 1-87).

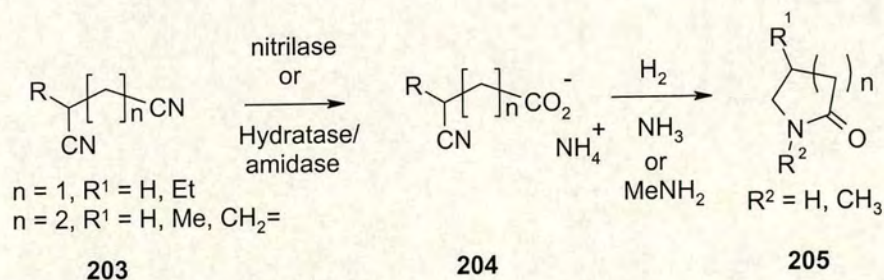


Fig 1-87 : Catalysed regioselective hydrolysis of aliphatic dinitriles.

The corresponding dicarboxylic acid di-ammonium salt was produced as a side-product but its formation was minimised by heating the suspension of the catalyst in buffer prior to addition of the substrate.



The regio- and stereo-selective hydrolysis of various aliphatic, aromatic nitriles and amides **206** has been elucidated in depth recently using resting cells of *Rhodococcus* sp. C3II and *Rhodococcus erythropolis* MP50 (Fig 1-88).<sup>133</sup> Both an alkyl and an aryl substituent in the  $\alpha$ -position was required for high enantioselectivity affording the (*S*)-acid with e.e. of 99 %.

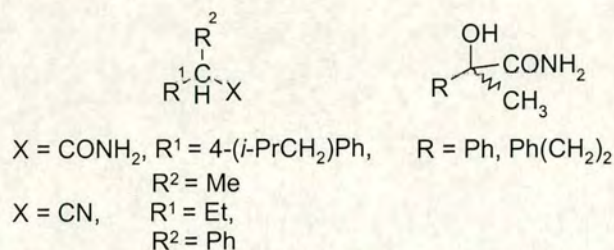
**206**

Fig 1-88 : Regioselective hydrolysis of nitriles and amides.

The same authors have reported on the regioselective hydrolysis of aromatic, aliphatic and unsaturated dinitriles **207** with these resting cells, which contain two enzymes, each causing nitrile-to-amide and amide-to-acid conversion (Fig 1-89).

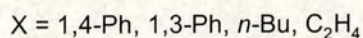
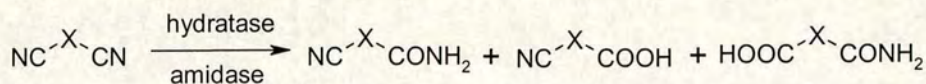
**207****208****209****210**

Fig 1-89 : Regioselective hydrolysis of nitriles using a combination of enzymes.

Both bacterial strains complemented each other due to the different activities and substrate preferences of the respective nitrile hydratases and amidases present in both systems. The *Rhodococcus* sp. C3II selectively afforded mononitrile/monoamide derivatives **208**, while *Rhodococcus erythropolis* MP50 preferentially produced mononitrile/mono-acid **209** and mono-acid/monoamide **210** derivatives.

Meth-Cohn and Wang have carried out an exhaustive study on the hydrolysis of dinitriles **211** using *Rhodococcus* sp. AJ270 (Fig 1-90).<sup>134</sup>



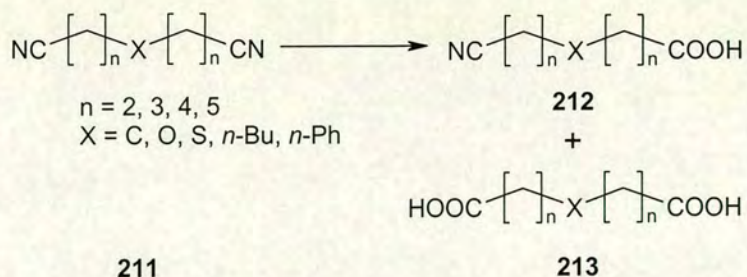


Fig 1-90 : *Rhodococcus* sp. AJ270 catalysed hydrolysis of dinitriles.

Their results showed that the mono-acid **212** was produced with those aliphatic dinitriles (dicyanoalkanes) which had short chains and the diacid **213** was preferred for longer chains. On inclusion of a heteroatom, the regioselectivity was dependent on its position in the chain. Mono-acids **212** were only produced in compounds with an oxygen atom  $\beta$ ,  $\gamma$  or  $\delta$  to the nitrile or with a sulfur atom in the  $\beta$  or  $\gamma$  position.

### 1.8.7 Chemical hydrolysis of nitriles/amides.<sup>103</sup>

Nitriles can be hydrolysed using traditional chemical methods with the most widely employed procedures involving strongly acidic, basic and/or oxidising ( $\text{H}_2\text{O}_2$ ) conditions. These methods have several drawbacks: the reaction has to be carried out under harsh reaction conditions and high temperatures which may not be compatible if other functionalities are present on the molecule. The formation of by-products such as toxic HCN or large amounts of salts pose a problem.

Compared with the chemical (non-enzymatic) route, the biotechnological route may have the following advantages: (a) less severe (mild) reaction conditions can be employed; (b) substrate and product specificity might be exhibited in cases where chemo-, stereo- and regio-selective transformations are difficult to achieve through non-enzymatic route; (c) formation of products with high purity; (d) the process is environmentally benign.

The mechanism for acid catalysed nitrile **164** hydrolysis is well known with the initial product formed being the amide **165** which is known to hydrolyse under these







## 2A. Results & Discussion : Synthesis of Potential Substrates.

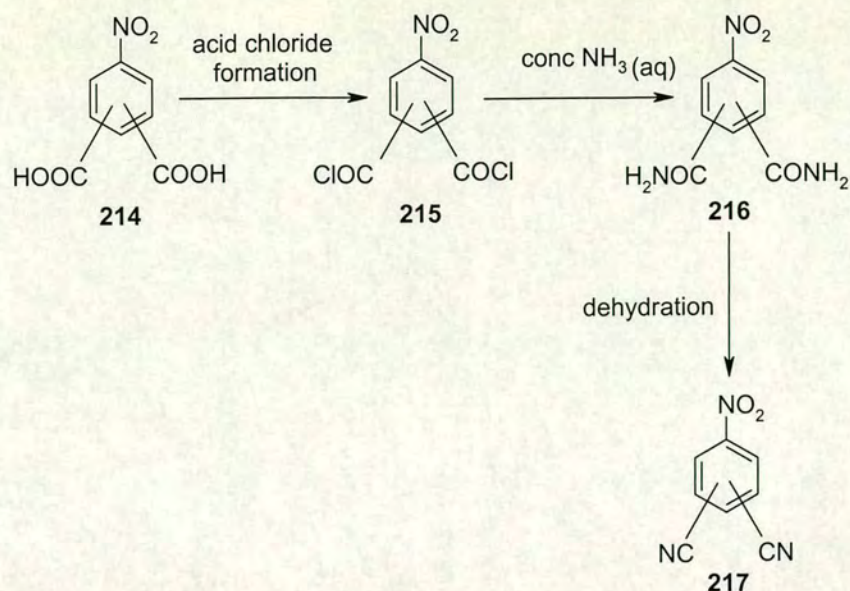
The first part of this chapter deals with the synthesis of a range of substrates (dicyanonitroarenes and (di)nitroarene-sulfinyls) for *S. cerevisiae* catalysed biotransformations. The attempted synthesis of dinitro-phthalonitrile has also been discussed.

### 2.1 Synthesis of dicyanonitroarenes.

To investigate the scope of *S. cerevisiae* catalysed nitroarene reduction, a range of nitroarenes with two cyano groups substituted around the ring were synthesised. Of the six possible isomers in which two cyano groups could be substituted on the aromatic ring along with the nitro group, two were commercially available.

Synthesis of the remaining four substrates was carried out in the following way. In general, the diacid **214** was converted into the diacid chloride **215** followed by conversion to the diamide **216** by the addition of aqueous ammonia solution. The dehydration of the diamide **216** was achieved by using phosphorus pentoxide or phosphorus oxychloride under different reaction conditions to afford crude dicyanonitroarenes **217** in moderate yields (Fig 2-1, Table 2-1).

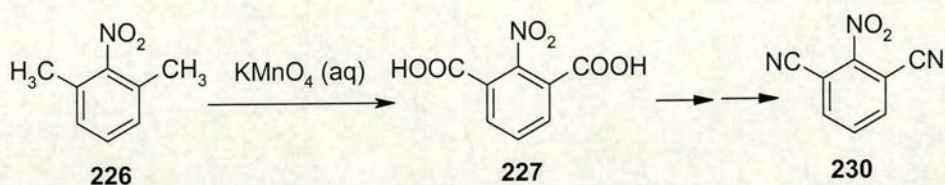


Fig 2-1 : Synthesis of dicyanonitroarenes **217**.

Diacid		Acid chloride		Diamide		Dicyanoarenes	
<b>218</b>	1,3-COOH, 5-NO <sub>2</sub>	97 %	<b>219</b>	56 %	<b>220</b>	50 %	<b>221</b>
<b>222</b>	1,4-COOH, 2-NO <sub>2</sub>	82 %	<b>223</b>	94 %	<b>224</b>	37 %	<b>225</b>
<b>227</b>	1,3-COOH, 2-NO <sub>2</sub>	25 %	<b>228</b>	96 %	<b>229</b>	67 %	<b>230</b>

Table 2-1 : Yields of products obtained in the synthesis of dicyanonitroarene.

In the case of 2-nitro-isophthalonitrile **230**, the diacid **227** was obtained from the oxidation of 1,3-dimethyl-2-nitrobenzene **226** by aqueous potassium permanganate (Fig 2-2).

Fig 2-2 : Synthesis of 2-nitro-isophthalonitrile **230**.

4-Nitro-isophthalonitrile **233** was produced by the permanganate oxidation of 3-methyl-4-nitro-benzoic acid **231** to afford the diacid **232** followed by reaction with



phosphorus pentachloride and *para*-toluenesulphonamide to yield the final product **233** in 22 % yield (Fig 2-3).

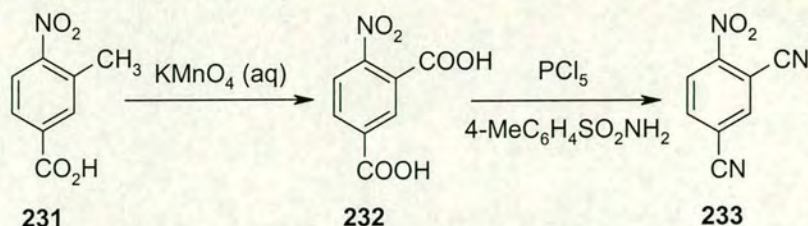


Fig 2-3 : Synthesis of 4-nitro-isophthalonitrile **233**.

## 2.2 Attempted synthesis of 3,6-dinitro-phthalonitrile **245**.

3-Nitro-phthalonitrile **238** (for the synthesis of 3,6-dinitro-phthalonitrile **245**) was used as a model study since it was similar in structure to **245**. The synthesis planned was analogous to the synthesis of dicyanonitroarenes (Fig 2-1), involving conversion of the diacid **234** into the diacid chloride **235**, followed by diamide **237** formation and then dehydration to afford 3-nitro-phthalonitrile **238**. If successful, this procedure could then be further applied to the synthesis of 3,6-dinitro-phthalonitrile **245** starting from the corresponding diacid **240**.

3-Nitrophthalic acid **234** was converted to the diacid chloride **235** in 78 % yield using thionyl chloride. However, a problem arose in the next step, instead of the desired diamide **237**, the corresponding imide **236** was obtained in a high yield of 73 % when the diacid chloride **235** was stirred with aqueous ammonia solution (Fig 2-4).

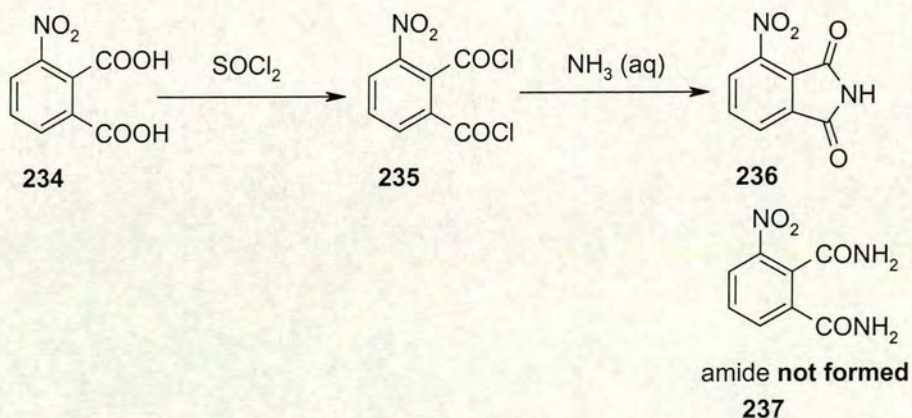
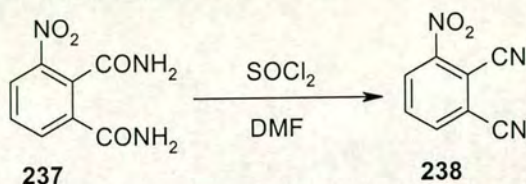


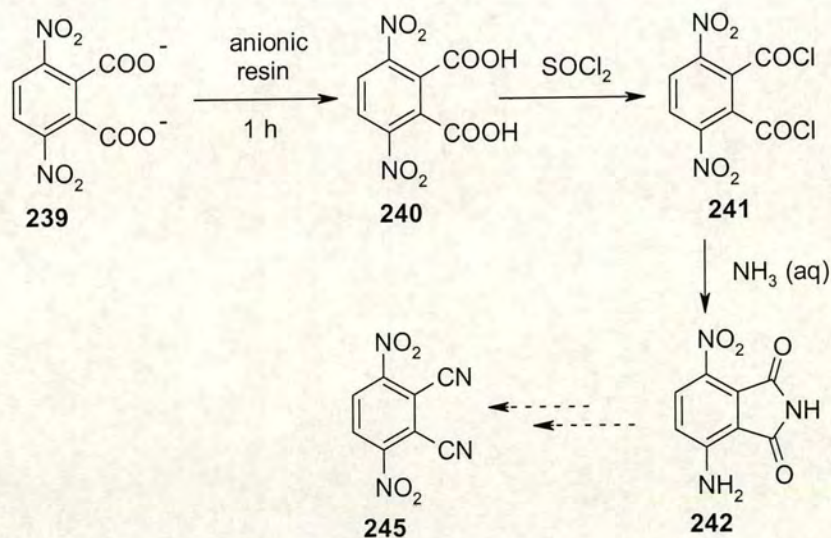


Fig 2-4 : Synthesis of 4-nitro-isoindole-1,3-dione **236**.

Subsequent treatment of the imide **236** in the minimum amount of concentrated aqueous ammonium hydroxide while stirring at 45 °C for 5 h followed by work-up afforded the diamide **237** in 62 % yield. Under anhydrous conditions the diamide **237** was then dehydrated using thionyl chloride in dimethylformamide to yield 3-nitro-phthalonitrile **238** in 72 % yield (Fig 2-5).

Fig 2-5 : Synthesis of 3-nitro-phthalonitrile **238**.

The above procedure was then applied to the synthesis of 3,6-dinitro-phthalonitrile **245**. The starting material 3,6-dinitro-phthalic acid pyridinium salt **239** was converted to the diacid **240** using anionic resin followed by treatment with thionyl chloride to afford the diacid chloride **241**, which was then stirred with aqueous ammonia solution to yield 4-amino-7-nitro-isoindole-1,3-dione **242** (Fig 2-6, Table 2-2) and not the corresponding imide **243** or diamide **244** which was expected (Fig 2-7).

Fig 2-6 : Synthesis of 4-amino-7-nitro-isoindole-1,3-dione **242**.



Product	Yield
<b>240</b>	92 %
<b>241</b>	75 %
<b>242</b>	73 %

Table 2-2 : Yields of products obtained in the synthesis of 4-amino-7-nitro-isoindole-1,3-dione **242**

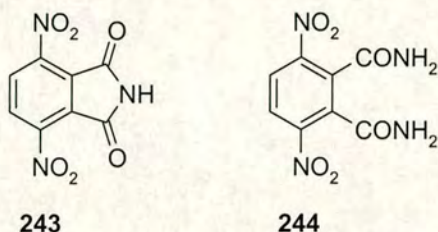


Fig 2-7 : Imide **262** and amide **263**.

It is believed that the presence of a *para*-nitro group and an *ortho*-electron withdrawing group on 3,6-dinitro-phthaloyl dichloride **241** activated the displacement of the other nitro group to afford **242** when stirred in aqueous ammonia solution. The presence of the amino group was evident from the mass spectrum data obtained, where a molecular ion with a molecular weight of 207 Da was recorded (Fig 2-8). Similar displacements of nitro groups in compounds that have cyano and *para*-electron withdrawing groups attached to the aromatic ring with various nucleophiles have been reported.<sup>137-139</sup>

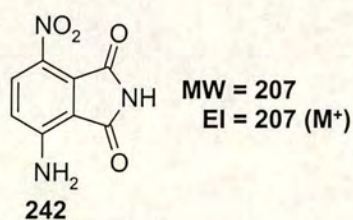


Fig 2-8 : 4-amino-7-nitro-isoindole-1,3-dione **242**.

Alternative reagents for diamide **244** formation from the diacid chloride **241** or even the diacid **240** were sought to prevent this exchange occurring in aqueous ammonia solution. 3-Nitro-phthaloyl dichloride **235** was then used as a model study and the problem with the nitro group displacement was partially solved by using



hexamethyldisilazide. However, only one acid chloride group in **235** was converted to the amide and the second remained intact to afford 2-carbamoyl-3-nitrobenzoyl chloride **246** (Fig 2-9). Stronger bases than hexamethyldisilazide were used but the desired diamide **237** product was not obtained. Evidence for the structure of **246** was provided by comparison of the expected<sup>140</sup> and observed frequency positions of the carbon atoms in **246** with those of its regioisomer **247**.

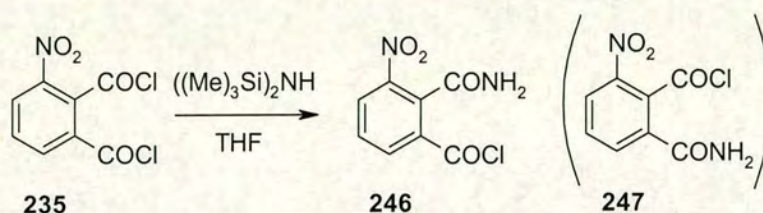


Fig 2-9 : Synthesis of 2-carbamoyl-3-nitrobenzoyl chloride **246**.

### 2.3 Synthesis of nitroarene-sulfinyls.

A range of substrates were synthesised which differed only by the substituent attached to the sulfur atom. The synthesis involved a two step process: the formation of the sulfanyl **248** followed by oxidation to the corresponding sulfinyl derivative **249** (Fig 2-10).

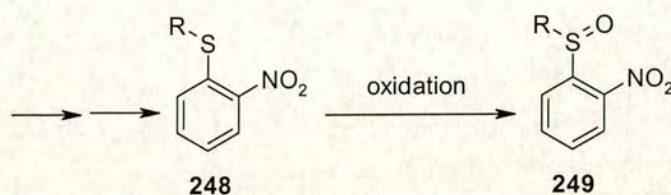
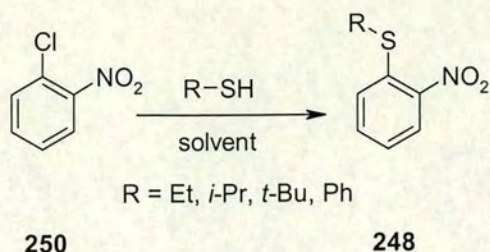


Fig 2-10 : General synthesis of nitroarene-sulfinyls **249**.

#### 2.3.1 Nitroarene-sulfanyl synthesis.

In general, the nitroarene-sulfanyls **248** were obtained from the reaction of *ortho*-chloro-nitrobenzene **250** with the corresponding thiol in different solvents and reaction conditions with moderate yields obtained (Fig 2-11, Table 2-3).

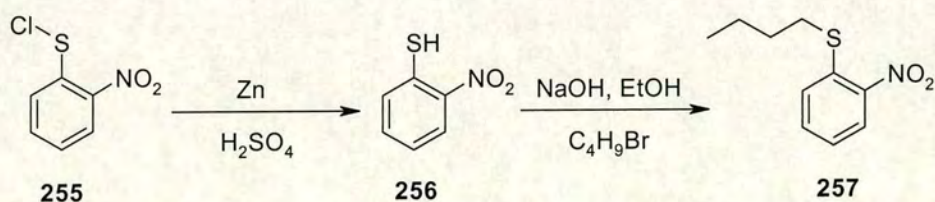


Fig 2-11 : Synthesis of nitroarene-sulfanyls **248**.

Thiol R-SH	Yield : sulfanyl (248) RSC <sub>6</sub> H <sub>4</sub> NO <sub>2</sub>	
Et	73 %	<b>251</b>
<i>i</i> -pr	84 %	<b>252</b>
<i>t</i> -Bu	42 %	<b>253</b>
Ph	11 %	<b>254</b>

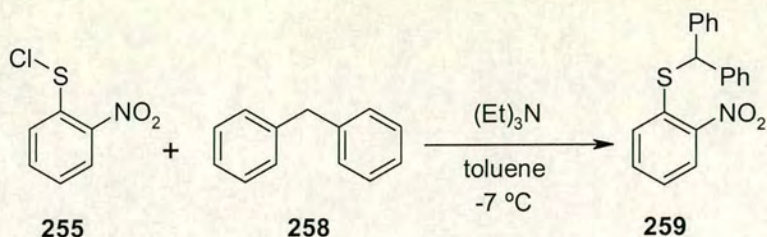
Table 2-3 : Yields of nitroarene-sulfanyls **248**.

*Ortho*-nitro-benzenethiol **256** was synthesised by the displacement of chloride from *ortho*-nitro-benzenesulfenyl chloride **255** by zinc metal in conc. sulfuric acid. Thereafter, bromobutane was added to a solution of *ortho*-nitro-benzenethiol **256** and NaOH in ethanol resulting in the displacement of the bromide in bromobutane to afford 1-butylsulfanyl-2-nitrobenzene **257** in 46 % yield (Fig 2-12).

Fig 2-12 : Synthesis of 1-butylsulfanyl-2-nitrobenzene **257**.

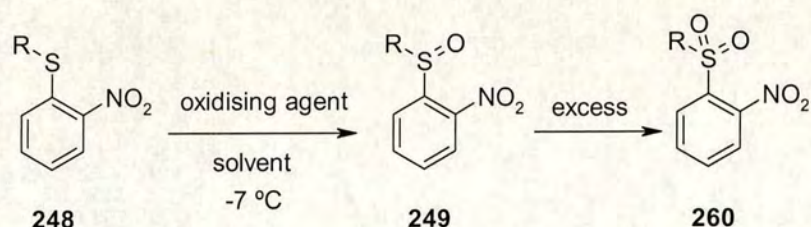
The synthesis of 1-benzhydrylsulfanyl-2-nitrobenzene **259** did not involve the use of thiol intermediates. Instead, triethylamine was added to a solution of *ortho*-nitro-benzenesulfenyl chloride **255** and diphenylmethane **258** in toluene resulting in the displacement of the chloride to afford the corresponding sulfanyl **259** in a low yield of 10 % (Fig 2-13).



Fig 2-13 : Synthesis of 1-benzhydrylsulfinyl-2-nitrobenzene **259**.

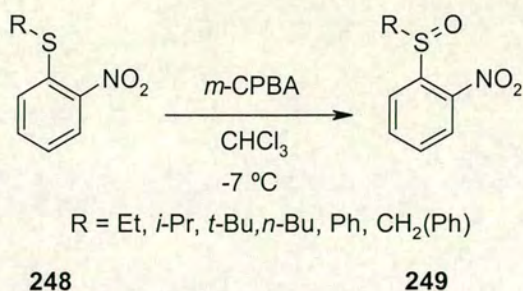
### 2.3.2 Nitroarene-sulfinyl synthesis.

In general, sulfanyls **248** could be oxidised to the corresponding sulfinyls **249** using either *meta*-chloroperoxybenzoic acid<sup>141</sup>, sodium *meta*-periodate<sup>141</sup> or urea-hydrogen peroxide (Fig 2-14).<sup>142</sup> A 1:1 ratio of oxidising agent to sulfanyl **248** was used to afford the sulfinyl **249** since excess of oxidant used results in formation of the sulfone **260**.

Fig 2-14 : Oxidation of sulfanyls to sulfinyls **250**.

Initially, urea-hydrogen peroxide was used to oxidise the sulfanyls to the sulfinyls because this was considered a safer alternative than *m*-CPBA, which may form explosive peroxides and could be considered dangerous over a period of time. However, the peroxide reagent failed to oxidise the sulfanyls to the corresponding sulfinyls and low yields of sulfinyls were obtained when sodium *meta*-periodate was used. When a slight excess of *m*-CPBA was used, however, the sulfanyls **248** were oxidised to the corresponding sulfinyls **249** in moderate yields (Fig 2-15, Table 2-4).



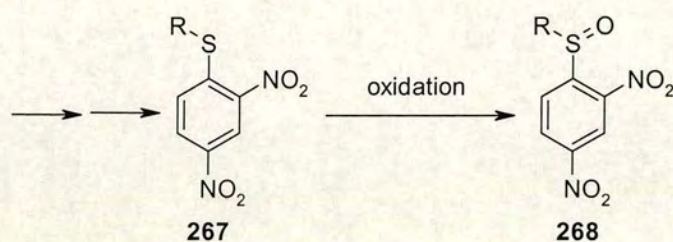
Fig 2-15 : Oxidation of nitroarene-sulfanyls to nitroarene-sulfinyls **249**.

Yield : sulfanyl (248)		Yield : sulfinyl (249)	
RSC <sub>6</sub> H <sub>4</sub> NO <sub>2</sub>		RS(O)C <sub>6</sub> H <sub>4</sub> NO <sub>2</sub>	
<b>251</b>	Et	99 %	<b>261</b>
<b>252</b>	<i>i</i> -pr	84 %	<b>262</b>
<b>253</b>	<i>t</i> -Bu	51 %	<b>263</b>
<b>254</b>	Ph	63 %	<b>264</b>
<b>257</b>	<i>n</i> -Bu	64 %	<b>265</b>
<b>259</b>	CH <sub>2</sub> (Ph) <sub>2</sub>	31 %	<b>266</b>

Table 2-4 : Yields of nitroarene-sulfinyls **249** obtained using *m*-CPBA.

## 2.4 Synthesis of dinitroarene-sulfinyls.

A range of substrates were synthesised which differed only by the substituent attached to the sulfur atom. The synthesis involved a two step process: the formation of the sulfanyl **267** followed by the oxidation to the corresponding sulfinyl derivative **268** (Fig 2-16).

Fig 2-16 : General synthesis of dinitroarene-sulfinyls **268**.



The synthesis of dinitroarene-sulfinyls was very similar to the nitroarene-sulfinyl synthesis (Section 2.3). The sulfanyls **267** were obtained by the reaction of *ortho*-chloro-2,4-dinitrobenzene **269** with the appropriate thiol. Subsequent oxidation of the sulfanyls **269** to the corresponding sulfinyls **268** in moderate yields was achieved using *m*-CPBA (Fig 2-17, Table 2-5).

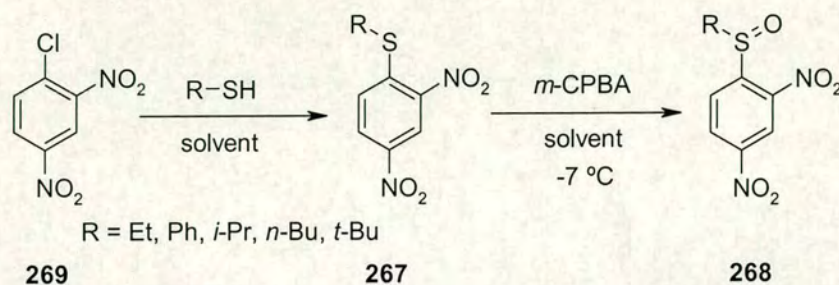


Fig 2-17 : Synthesis of dinitroarene-sulfinyls **268**.

Thiol R-SH	Yield : sulfanyl (267) RSC <sub>6</sub> H <sub>4</sub> (NO <sub>2</sub> ) <sub>2</sub>		Yield : sulfinyl (268) RS(O)C <sub>6</sub> H <sub>3</sub> (NO <sub>2</sub> ) <sub>2</sub>	
Et	19 %	<b>270</b>	63 %	<b>275</b>
<i>i</i> -Pr	62 %	<b>271</b>	21 %	<b>276</b>
<i>t</i> -Bu	35 %	<b>272</b>	-----	
Ph	34 %	<b>273</b>	54 %	<b>277</b>
<i>n</i> -Bu	32 %	<b>274</b>	68 %	<b>278</b>

Table 2-5 : Yields of dinitroarene-sulfanyls **267** and sulfinyls **268**.

Different reaction conditions were examined in an attempt to oxidise *tert*-butylsulfanyl-2,4-dinitrobenzene **272** to the corresponding sulfinyl but the starting material was recovered in quantitative yield in all cases.



The synthesis of benzhydrylsulfanyl-2,4-dinitrobenzene **280** did not involve the use of thiol intermediates. Instead, triethylamine was added to a solution of 2,4-dinitrobenzenesulfonyl chloride **279** and diphenylmethane **258** in toluene to afford the corresponding sulfanyl **280** in 42 % yield (Fig 2-18). Oxidation of the sulfanyl **280** using *m*-CPBA afforded 1-(diphenyl-methanesulfinyl)-2,4-dinitrobenzene **281** in 41 % yield.

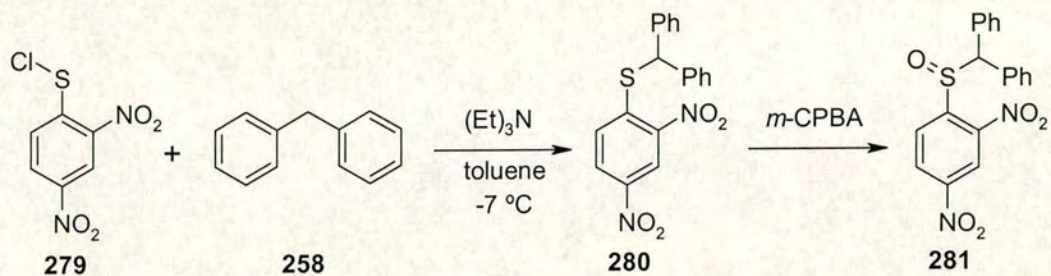


Fig 2-18 : Synthesis of 1-(diphenyl-methanesulfinyl)-2,4-dinitrobenzene **281**.



## 2B. Results & Discussion : Biotransformation of Substrates.

The second part of this chapter describes *S. cerevisiae* catalysed nitro group reduction of the substrate types described in Chapter 2A as well as others which were commercially available. Initially, the reduction of (di)cyanonitroarenes is discussed followed by (di)nitroarene-sulfinyl reduction.

### 2.5 Optimised conditions for *S. cerevisiae* catalysed reduction.

GCMS was used to monitor the progress of the biotransformation of (di)cyanonitroarenes. Chiral HPLC and reverse-phase HPLC were employed for the progress of reduction of (di)nitroarene-sulfinyl substrates. The optimised method reported by Blackie *et al.*<sup>66</sup> was employed to obtain clean and reasonable yields of the reduced products (Fig 2-19) and *S. cerevisiae* was pre-washed with acetone at -20 °C prior to its use. Using this procedure, the biotransformations were found to be much cleaner with fewer impurities present.

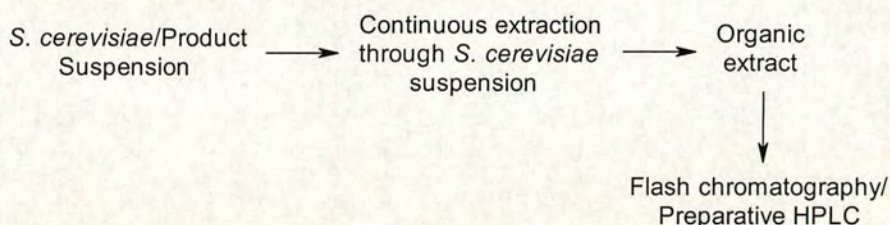


Fig 2-19 : Optimised method for *S. cerevisiae* catalysed reduction.



## 2.6 Microbial and chemical reduction of 2-nitro-benzonitrile **282**.

### 2.6.1 *S. cerevisiae* catalysed reduction of 2-nitro-benzonitrile **282**.

Previous studies on the reduction of 2-nitro-benzonitrile **282** using *S. cerevisiae* afforded the expected 2-amino-benzonitrile **283** product.<sup>3</sup> But, Davey *et al.* reported on this reduction to give an unexpected result, with the formation of 2-amino-benzamide **284** as a major product and the expected 2-amino-benzonitrile **283** as a minor product in the biotransformation (Fig 2-20).<sup>2</sup>

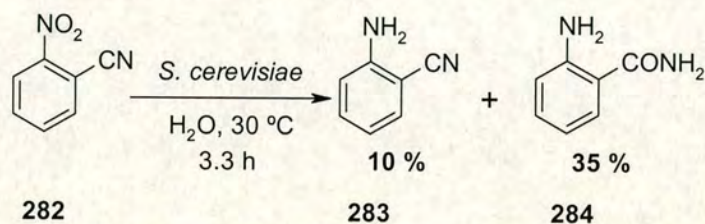


Fig 2-20 : *S. cerevisiae* catalysed reduction of 2-nitro-benzonitrile **282**.

### 2.6.2 Chemical reduction of 2-nitro-benzonitrile **282**.

In 1895, Pinnow *et al.* reported on the chemical reduction of 2-nitro-benzonitrile **282** under various reducing conditions. Interestingly, the addition of cooled ( $-10\text{ }^\circ\text{C}$ ) acetic acid and water to **282** followed by the addition of zinc powder resulted in the formation of 2-amino-benzamide **284** and a side product which was not then identified (Fig 2-21).<sup>143</sup>

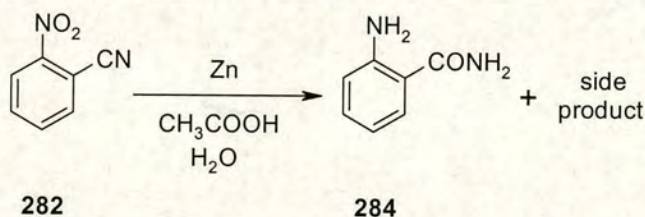


Fig 2-21 : Chemical reduction of 2-nitro-benzonitrile **282**.

A recent paper has reported on the formation of the expected 2-amino-benzonitrile product **283** using nickel in *iso*-propanol followed by the addition of 2-nitro-benzonitrile **282** with 100 % conversion to **283** (Fig 2-22).<sup>144</sup>



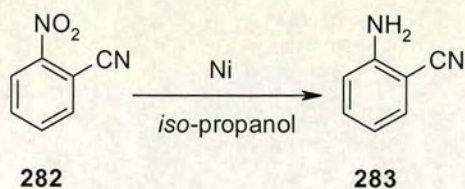


Fig 2-22 : Chemical reduction of 2-nitro-benzonitrile **282**.

### 2.6.3 Conclusion.

It appears that *S. cerevisiae* catalysed reduction and chemical reduction of 2-nitro-benzonitrile **282** afford similar products, 2-amino-benzamide **284** and 2-amino-benzonitrile **283**. Therefore, similar mechanism and intermediate(s) may be involved in both processes. The chemical reduction of a nitro group to the corresponding aniline is known to proceed *via* a series of two electron additions to form a nitroso derivative and hydroxylamine.<sup>67</sup> Intermediates similar in structure to the nitroso and hydroxylamine derivatives may be involved in the microbial reduction of 2-nitro-benzonitrile **282** which is further discussed in Chapter 4.

## 2.7 Reduction of cyanonitroarenes.

The reduction of the nitro group to the expected amino product was also observed to occur when the cyano group was substituted *meta* **285** or *para* **287** to the nitro group on the aromatic ring. However, the cyano group remained intact to afford the reduced products **286**, **288** in moderate yields (Fig 2-23, Fig 2-24).

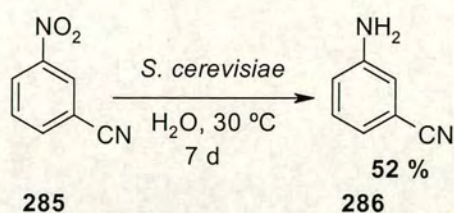


Fig 2-23 : *S. cerevisiae* catalysed reduction of 3-nitro-benzonitrile **285**.



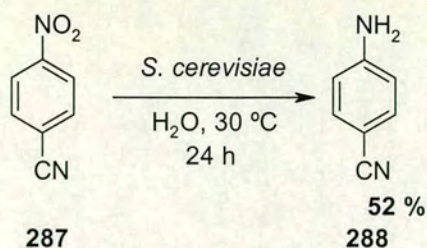


Fig 2-24 : *S. cerevisiae* catalysed reduction of 4-nitro-benzonitrile **287**.

This protocol was extended further to investigate the reduction of dicyanonitroarenes by *S. cerevisiae* to determine whether similar products would be formed.

## 2.8 Reduction of dicyanonitroarenes.

Incubation of 3-nitro-phthalonitrile **238** with *S. cerevisiae* resulted in the reduction of the nitro group and the conversion of the *ortho* cyano group to the amide moiety **289** in a yield of 50 % and 2-amino-6-cyano-benzoic acid **290** was obtained in a low yield of 10 % (Fig 2-25).

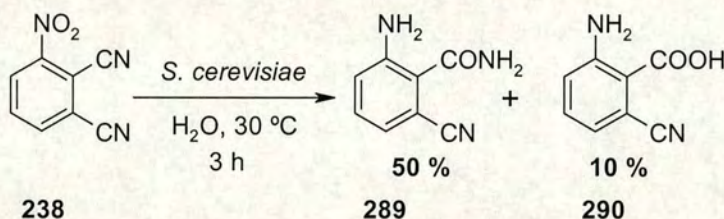


Fig 2-25 : *S. cerevisiae* catalysed reduction of 3-nitro-phthalonitrile **238**.

The regioselectivity was investigated in substrates similar in structure to 3-nitro-phthalonitrile **238**. The first class of compounds **225**, **230**, **233** consisted of one cyano group substituted *ortho* to the nitro group on the aromatic ring and the other cyano group at either 4, 5 or 6 position to the nitro group (Fig 2-26).



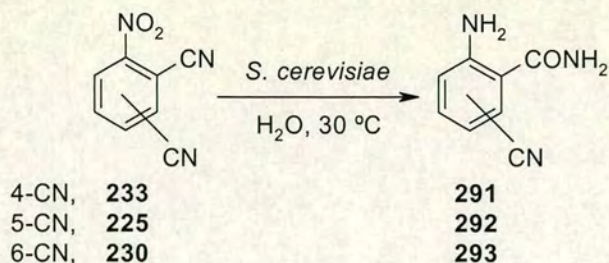


Fig 2-26 : *S. cerevisiae* catalysed reduction of dicyanonitroarenes.

Reduction of the nitro group occurred as expected and the selective conversion of the *ortho* cyano group was observed to afford the amide products **291**, **292**, **293** in moderate yields (Table 2-6).

Substrate		Reaction Time	Yield
<b>233</b>	<b>4-CN</b>	30 min	62 % <b>291</b>
<b>225</b>	<b>5-CN</b>	45 min	62 % <b>292</b>
<b>230</b>	<b>6-CN</b>	30 min	74 % <b>293</b>

Table 2-6 : Yields and reaction times of reduced products obtained.

For the reduction of 2-nitro-isophthalonitrile **230**, where both cyano groups were substituted *ortho* to the nitro group on the ring, only one group was selectively converted to the amide and the other remained intact to afford **293** in 74 % yield (Fig 2-27).

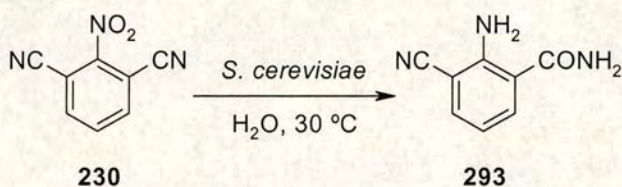


Fig 2-27 : *S. cerevisiae* catalysed reduction of 2-nitro-isophthalonitrile **230**.

The reduction of the nitro group to the expected amino products **295**, **296** occurred in the substrates **221**, **294** that had no cyano groups substituted *ortho* to the nitro group on the ring (Fig 2-28). Generally, the reaction times were longer compared to those



isomeric substrates which had a cyano group substituted *ortho* to the nitro group on the ring (Table 2-7).

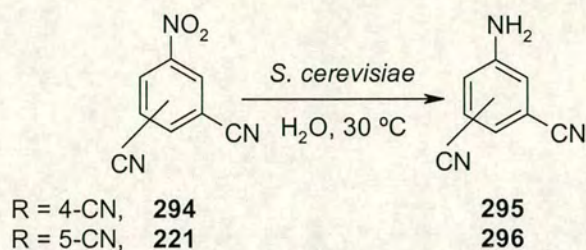


Fig 2-28 : *S. cerevisiae* catalysed reduction of dicyanonitroarenes.

Substrate	Reaction Time	Yield
<b>294</b> 4-CN	2 h	46 % <b>295</b>
<b>221</b> 5-CN	7 h	76 % <b>296</b>

Table 2-7 : Yields and reaction times of reduced products obtained.

## 2.9 2-Amino-6-cyano-benzoic acid **290** formation.

2-Amino-6-cyano-benzoic acid **290** (Fig 2-29) was produced in the reduction of 3-nitro-phthalonitrile **238**, whereas no acid product was detected in the reduction of the other dicyanonitroarenes **225**, **230**, **233** previously mentioned.

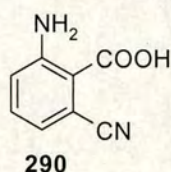


Fig 2-29 : 2-Amino-6-cyano-benzoic acid **290**.

A series of control experiments were carried out to determine whether the acid **290** was a result of the biotransformation or a side product. Firstly, 2-amino-6-cyano-benzamide **289** was incubated with *S. cerevisiae* with the progress monitored by GCMS. The acid **290** was detected over a period of time as the concentration of the amide **289** decreased (Table 2-8). Similarly, the addition of the amide **289** to water



resulted in the formation of the acid **290** whereas the starting material was recovered in quantitative yield when the acid **290** was incubated with *S. cerevisiae*.

Substrate	Reaction Conditions	Product
<b>289</b> Amide	Incubation with <i>S. cerevisiae</i>	Acid <b>290</b>
<b>289</b> Amide	Addition to water	Acid <b>290</b>
<b>290</b> Acid	Incubation with <i>S. cerevisiae</i>	Starting material

Table 2-8 : Controlled experiments.

It was evident that 2-amino-6-cyano-benzoic acid **290** was a result of the spontaneous hydrolysis of 2-amino-6-cyano-benzamide **289** which was produced when 3-nitro-phthalonitrile **238** was incubated with *S. cerevisiae* (Fig 2-30).

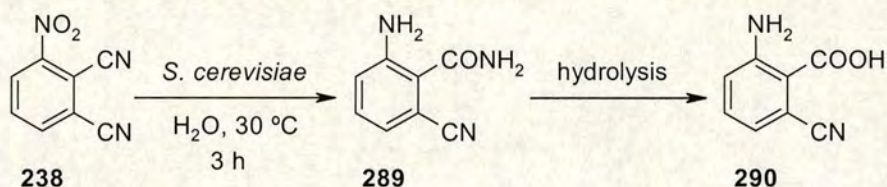


Fig 2-30 : *S. cerevisiae* catalysed reduction of 3-nitro-phthalonitrile **238**.

In contrast, no acid formation occurred with the other amide products **291**, **292**, **293** when incubated under the same conditions. It is possible that the presence of the cyano group *ortho* to the amide moiety on the ring in 2-amino-6-cyano-benzamide **289** plays a significant role which leads to the hydrolysis of the amide group to afford **290**.

## 2.10 Conclusion.

The cyano group underwent selective conversion to the amide when it was substituted *ortho* to the nitro group on the aromatic ring in (di)cyanonitroarene substrates, whereas the *meta* or *para* substituted cyano group(s) remained intact. Therefore this provides a basis for selectively converting and manipulating one



cyano group in the presence of another. Dicyanonitroarene substrates underwent reduction faster than those substrates that had only one cyano group substituted on the ring.

### 2.11 Reduction of 3,6-dinitro-phthalonitrile **245**.

The aim was to chemically synthesise 3,6-dinitro-phthalonitrile **245** and test it as a substrate for *S. cerevisiae* reduction. If the substrate was transformed, one possibility for the reduced product could be a 1,2,3,4-tetra-substituted aromatic ring **297** where all 4 functional groups are different (Fig 2-31). This would result from the reduction of one of the nitro groups (which is expected) and also selective conversion of one cyano group.

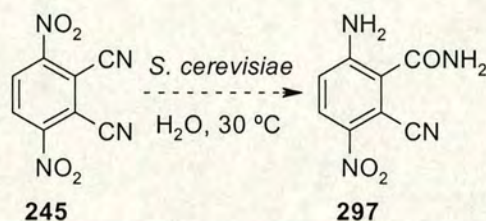


Fig 2-31 : *S. cerevisiae* catalysed reduction of 3,6-dinitro-phthalonitrile **245**.

The reduction of one of the nitro groups and conversion of one of the cyano groups would be very difficult to achieve under chemical conditions, since it would be difficult to distinguish between the two nitro and cyano groups. This may be possible to achieve under biological conditions since *S. cerevisiae* is known to reduce one of the nitro groups in 1,4-dinitrobenzene and regioselectively convert the cyano group substituted *ortho* to the nitro group on the ring in dicyanonitroarene substrates.

However, numerous attempts at the synthesis of the symmetrical substrate **245** failed (Section 2.2), so it was not possible to determine what the reduced product(s), if any, of this biotransformation would have been. The synthesis followed was analogous to the synthesis of dicyanonitroarenes (Fig 2-1) but a problem arose, instead of the desired diamide **244**, 4-amino-7-nitro-isindole-1,3-dione **242** was obtained from the reaction of 3,6-dinitro-phthaloyl dichloride **241** with aqueous ammonia solution. It



appeared that a nitro group was displaced from the diacid chloride **241** to afford **242**. Alternative reagents were sought to prevent this exchange to form the diamide **244** but only one acid chloride group was converted to the amide and the other remained intact.

If the synthesis and biotransformation was possible then this would have been a good example of using whole-cell systems over chemical methodology by going from a symmetrical structure to a product where all 4 functional groups were different.

## 2.12 Reduction of nitroarene-sulfinyls.

The aim of this part of the project was to investigate whether *S. cerevisiae* could catalyse the enantioselective reduction of nitroarene-sulfinyls **249** to afford optically active **298**, **299** (Fig 2-32).

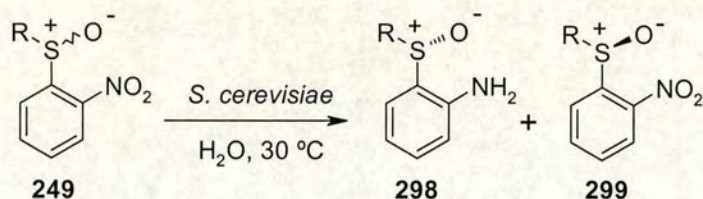


Fig 2-32 : *S. cerevisiae* catalysed enantioselective reduction of nitroarene-sulfinyls **249**.

Previous work carried out on the reduction of nitroarene-sulfinyls **300** resulted in product(s) formation which rapidly decomposed, making it difficult to determine any enantioselectivity in the process (Fig 2-33).<sup>66</sup>

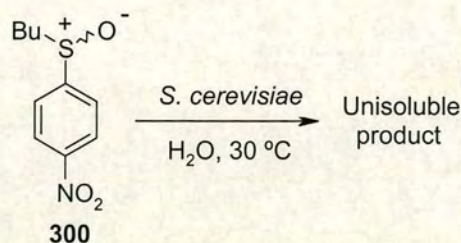


Fig 2-33 : *S. cerevisiae* catalysed reduction of butyl-4-nitro-phenylsulfinyl **300**.



We reasoned that by making the substituent attached to the sulfur atom larger and bulkier and by positioning the nitro group *ortho* instead of *para* to the sulfur on the ring, the chances of obtaining enantioselective reduction might be increased.

A series of nitroarene-sulfinyl substrates were incubated with *S. cerevisiae* and the progress of the reduction was monitored by chiral HPLC to determine the enantioselectivity of the reaction. Both enantiomers of the racemic sulfinyls **249** underwent reduction to the corresponding racemic amino-sulfinyl product(s) **301** with no evidence of enantioselectivity (Fig 2-34).

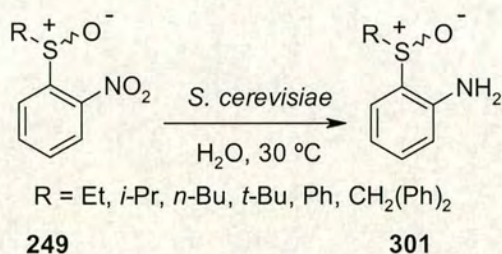


Fig 2-34 : *S. cerevisiae* catalysed reduction of nitroarene-sulfinyls **249**.

The substrates underwent reduction in short reaction times (with the exception of 1-(benzenesulfinyl)-2-nitrobenzene **264**) with moderate yields of the reduced products obtained which decomposed over a period of time (Table 2-9).

Sulfinyl (249)		Reaction time	Yield : Amino-sulfinyl (301)	
R			RS(O)C <sub>6</sub> H <sub>4</sub> NH <sub>2</sub>	
<b>261</b>	<b>Et</b>	6 h	62 %	<b>302</b>
<b>262</b>	<b><i>i</i>-pr</b>	6 h	45 %	<b>303</b>
<b>263</b>	<b><i>t</i>-Bu</b>	5 h	49 %	<b>304</b>
<b>264</b>	<b>Ph</b>	3 d	45 %	<b>305</b>
<b>265</b>	<b><i>n</i>-Bu</b>	7 h	55 %	<b>306</b>
<b>266</b>	<b>CH<sub>2</sub>(Ph)<sub>2</sub></b>	24 h	56 %	<b>307</b>

Table 2-9 : Yields and reaction times of racemic amino-sulfinyl products.



The biotransformations were monitored at regular time intervals by chiral HPLC using ethanol/hexane as the mobile phase. The progress of reduction of benzenesulfinyl-2-nitrobenzene **264** is shown (Fig 2-35 i-iv).

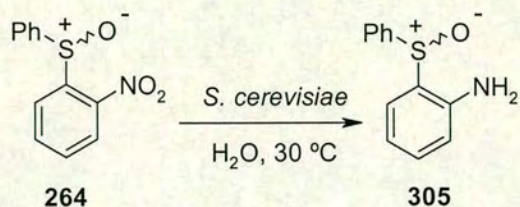


Fig 2-35 : *S. cerevisiae* catalysed reduction of benzenesulfinyl-2-nitrobenzene **264**.

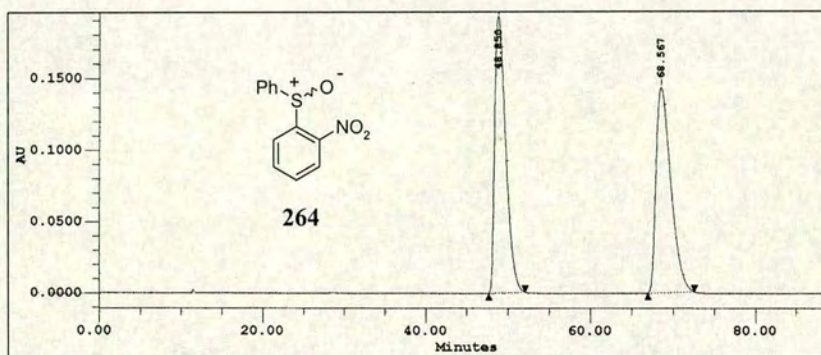


Fig 2-35i : Standard : benzenesulfinyl-2-nitrobenzene **264**.

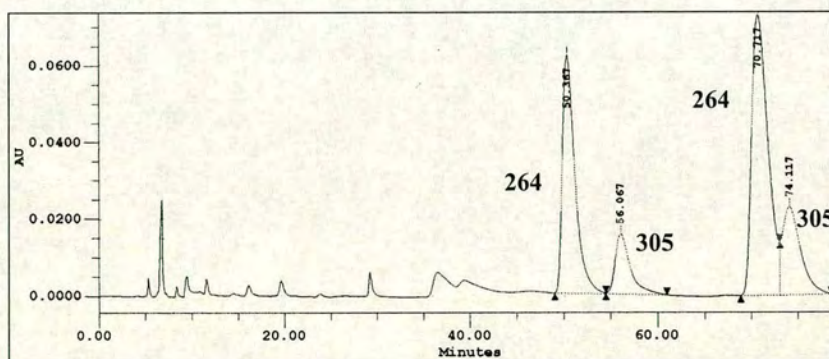


Fig 2-35ii : Reaction time = 1 h.



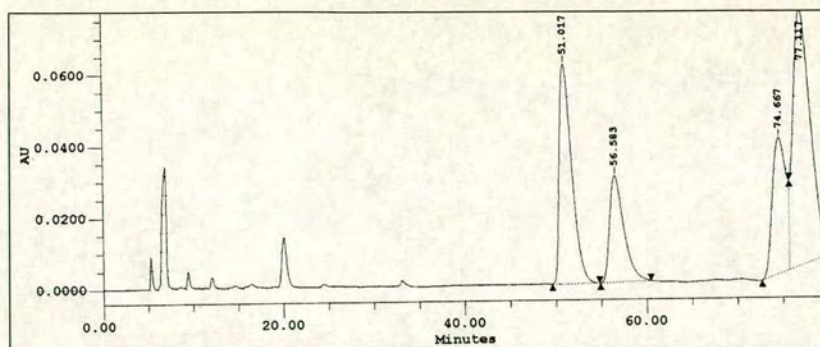


Fig 2-35iii : Reaction time = 24 h.

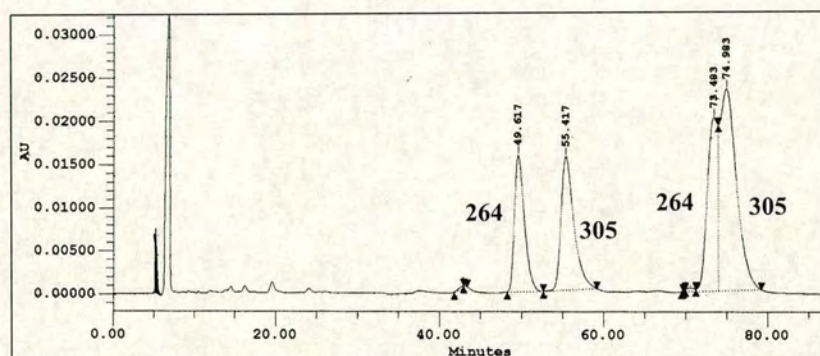


Fig 2-35iv : Reaction time = 3 d.

Thus it appears that reduction of both enantiomers of the racemic sulfinyl **264** occurred to form the racemic amino-sulfinyl **305**.

### 2.13 Reduction of dinitroarene-sulfinyls.

Investigations into possible enantioselective reduction of nitroarene-sulfinyls was extended one step further by using dinitroarene-sulfinyls as substrates. In the reduction of dinitroarene-sulfinyls **268**, four isomers can be produced, *i.e.* 2 regioisomers **308**, **309**, each of which exists as a pair of enantiomers (Fig 2-36). A range of 2,4-dinitroarene-sulfinyl substrates **268** were incubated with *S. cerevisiae* with the progress of reduction monitored by chiral HPLC.



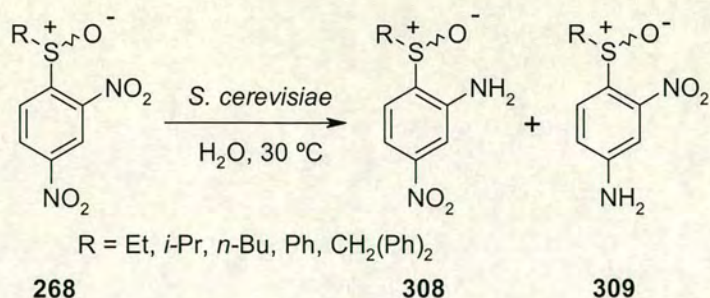


Fig 2-36 : *S. cerevisiae* catalysed regioselective reduction of dinitroarene-sulfinyls **268**.

Both enantiomers of the racemic sulfinyls **268** underwent reduction at both nitro groups relatively in the same proportion to each other, thus displaying no regioselectivity (Table 2-10). The amino-nitroarene-sulfinyl products **308**, **309** were produced in short reaction times but decomposed over a period of time.

Sulfinyl (268)		Reaction time	Yield : Amino-nitro-sulfinyl			
R			RS(O)C <sub>6</sub> H <sub>3</sub> (NH <sub>2</sub> )(NO <sub>2</sub> )			
			<i>Ortho</i> (308)		<i>Para</i> (309)	
275	Et	4 h	33 %	310	30 %	311
276	<i>i</i> -pr	3 h	30 %	312	29 %	313
277	Ph	2 h	36 %	314	32 %	315
278	<i>n</i> -Bu	4 h	37 %	316	31 %	317
281	CH <sub>2</sub> (Ph) <sub>2</sub>	3 h	33 %	318	31 %	319

Table 2-10 : Yields and reaction times of amino-nitroarene-sulfinyls **308**, **309**.

The individual regioisomers were characterised spectroscopically by <sup>1</sup>H NMR nOe studies, as shown for ethanesulfinyl-2,4-dinitrobenzene **310**, **311** (Fig 2-37). The irradiation of the proton of the amino group signal in the <sup>1</sup>H NMR spectrum of **310** caused an enhancement of H<sup>1</sup>. Subsequent irradiation of the amino group of **311** caused enhancement of both H<sup>1</sup> and H<sup>2</sup>. The other reduced amino-nitroarene-sulfinyl products obtained were similarly characterised.



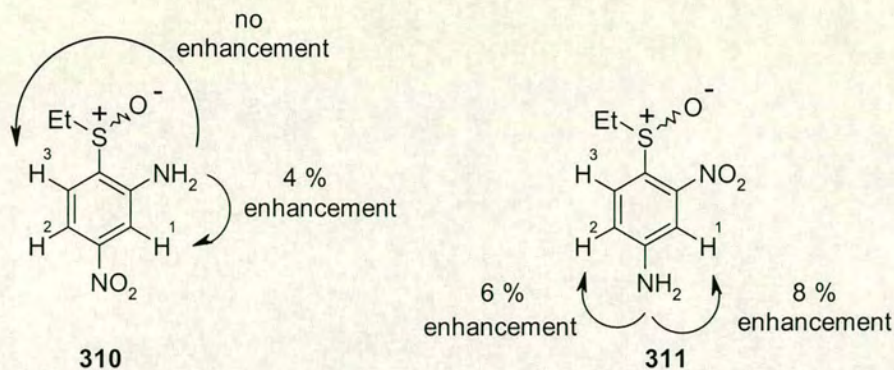


Fig 2-37 :  $^1\text{H}$  NMR nOe enhancements caused by irradiation of the amino group resonance.

## 2.14 Conclusion.

*S. cerevisiae* did not display any enantio- or regio-selectivity towards (di)nitroarene-sulfinyls. However, the reduction of the nitro group was observed to afford the corresponding amino-sulfinyl products which decomposed over a period of time. These findings suggested that these aromatic sulfinyls were excellent substrates for reduction catalysed by *S. cerevisiae* even though no stereoselectivity was displayed.



### 3. Results & Discussion : Nitro-Reductase Studies.

This chapter describes work directed towards the reduction of simple nitro aromatic compounds using nitro-reductases which have been isolated from *S. cerevisiae*. Other enzymes present in *S. cerevisiae* have also been investigated for their reducing activity.

#### 3.1 Isolated enzymes from *S. cerevisiae*.

*S. cerevisiae* catalysed reduction was investigated in a range of nitroarene substrates, so it was important to identify and characterise the reductase enzymes present in *S. cerevisiae* that were specific for this nitro group reduction in simple nitro aromatic compounds.

In our laboratories, Marina Alexeeva has isolated and purified three enzymes from *S. cerevisiae* which were shown to have the activity to reduce the nitro group to the corresponding amino moiety in simple nitroarenes.<sup>94</sup> A SDS-PAGE gel shows the relative purified bands of proteins with their corresponding molecular weights against a marker with known molecular weight (Fig 3-1).

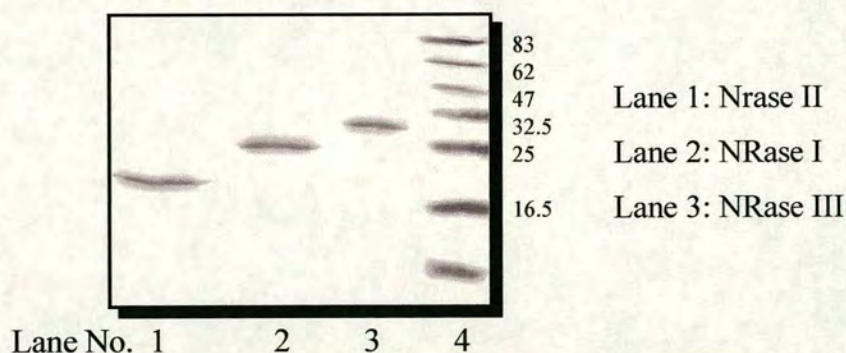


Fig 3-1 : Bands of the purified proteins against a marker.



The three purified proteins were identified as:

- Thioredoxin reductase (NRase I)
- NAD(P)H dehydrogenase, Old Yellow Enzyme II (OYE II) (NRase II)
- Lipoamide dehydrogenase (NRase III)

Studies were carried out using the 3 purified nitro-reductases and also some other isolated enzymes:

- Glutathione reductase which is present in *S. cerevisiae*.
- L-Lactate dehydrogenase which is present in *S. cerevisiae*.
- P-450 Cytochrome reductase which is present in *S. cerevisiae*, but this specific strain of enzyme used was obtained from *Pascillus bacterium*.

Reaction conditions were optimised so that the enzymes reduced the maximum amount of substrate with the minimum concentration required. Isolated enzyme systems require co-factors, NADPH or NADH, which work as electron donors in the reduction process were used to determine the maximum efficiency of each enzyme.

Generally, the substrate was incubated with each enzyme over a period of 1 h or 2 h. The progress of the reduction of the substrate was monitored by GCMS with samples withdrawn at fixed time intervals of 1 h and/or 2 h from the reaction mixture. The results obtained are summarised as graphs of reaction time versus percentage conversion to product, with respect to the disappearance of the starting material.

The graphs represented in this chapter are indications of what % of product is present after an x amount of time and no errors or whatsoever have been taken into account or calculated.

### **3.2 Reduction of dinitrobenzene 320.**

The reduction of 1,4-dinitrobenzene **320** by *S. cerevisiae* proceeded with selective reduction of one nitro group to afford 4-nitro-aniline **321** in 45 % yield (Fig 3-2). The same product **321** was obtained when 1,4-dinitrobenzene **320** was incubated



with the isolated enzymes, with each enzyme displaying different activity towards the nitro group.

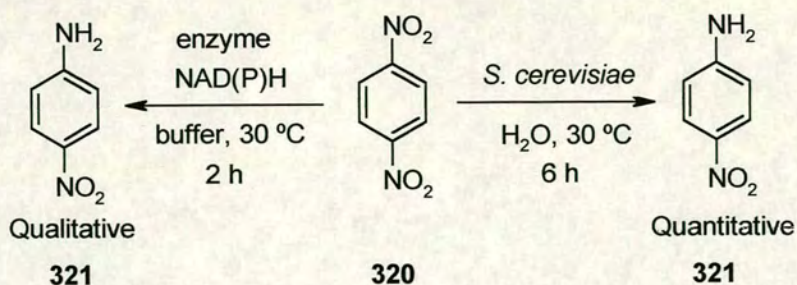
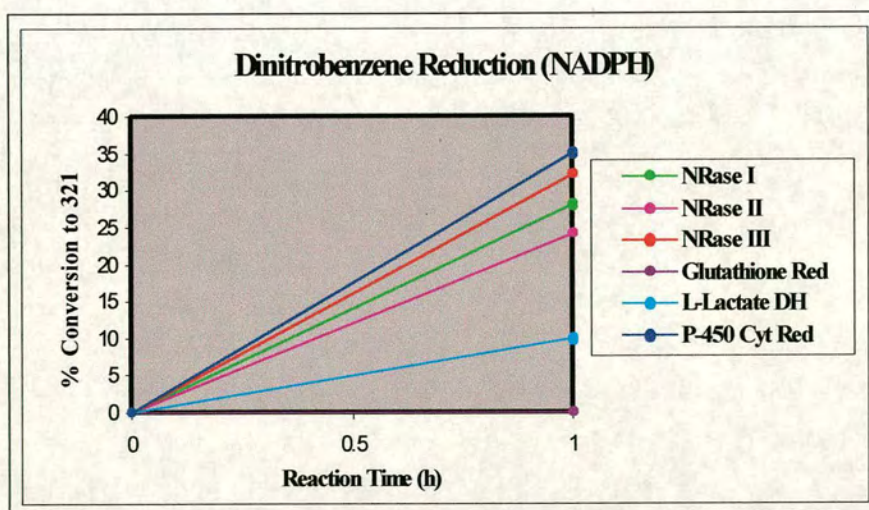


Fig 3-2 : Reduction of 1,4-dinitrobenzene **320**.

### 3.2.1 Reduction of dinitrobenzene **320** using NADPH.

The substrate **320** was dissolved in the minimum amount of DMSO and added to a solution of the enzyme(s) and NADPH in tris buffer. The reduction of the substrate was monitored by GCMS with a sample withdrawn at 1 h from the reaction mixture. The results obtained are plotted in the form of Graph 3-1.



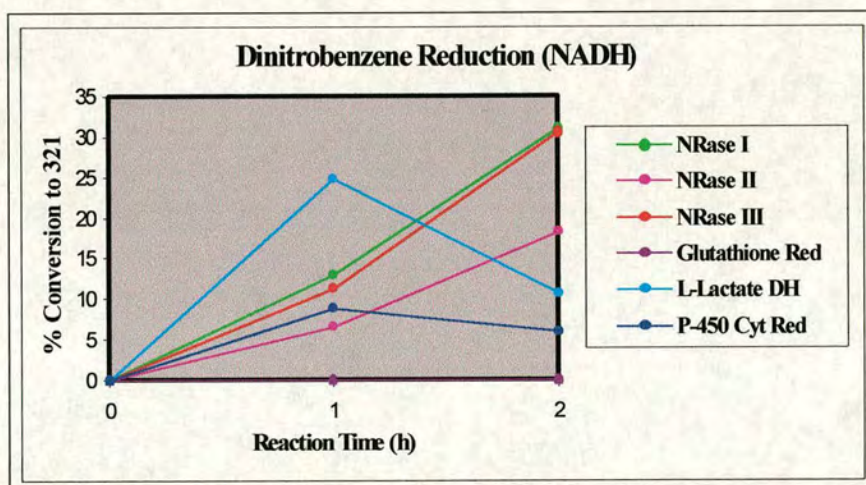
Graph 3-1 : Reduction of dinitrobenzene **320** using isolated enzymes.



P-450 cytochrome reductase, NRase I, II and III were the most effective in terms of percentage conversion to 4-nitro-aniline **321** over a period of time. Glutathione reductase failed to display any reducing activity towards the nitro group.

### 3.2.2 Reduction of dinitrobenzene **320** using NADH.

The substrate **320** was dissolved in the minimum amount of DMSO and added to a solution of the enzyme(s) and NADH in tris buffer. The reduction of the substrate was monitored by GCMS with samples withdrawn at regular time intervals (1 h, 2h) from the reaction mixture. The results obtained are plotted in the form of Graph 3-2.



Graph 3-2 : Reduction of dinitrobenzene **320** using isolated enzymes.

NRase I, II and III showed the maximum reducing activity when NADH was used over 2 h. Glutathione reductase displayed no reducing activity towards the nitro group.

It is well documented that the reduction of the nitro group in simple nitroarene compounds **320** to the corresponding amino derivative **321** by *S. cerevisiae* may proceed through nitroso **322** and hydroxy **323** derivatives (Fig 3-3).<sup>69</sup>



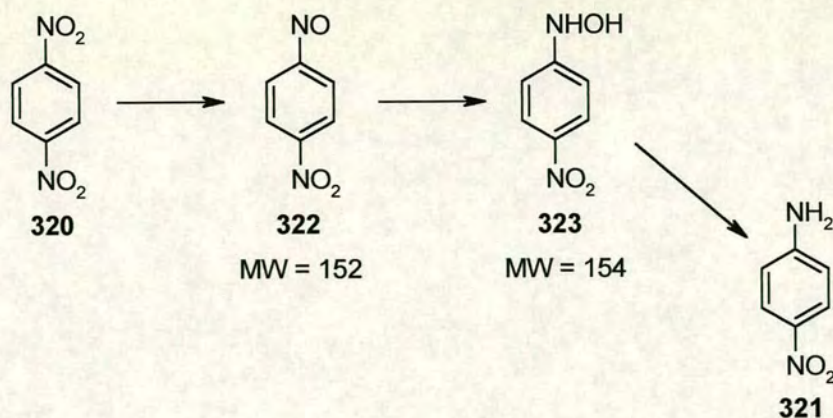


Fig 3-3 : Proposed mechanism for *S. cerevisiae* catalysed reduction of dinitrobenzene **320**.

An intermediate peak was recorded at 1 h reaction time in those biotransformations with isolated enzymes which afforded 4-nitro-aniline **321** as the product. The peak was scanned by the EI mass detector and displayed a molecular ion with a molecular weight of 154 Da. The proposed intermediate, nitro-phenyl hydroxylamine **323** has a molecular weight of 154 which suggests that hydroxylamine type of derivatives may be intermediates in the reduction pathway. Details of the intermediate(s) involved in the reduction process will be discussed more fully in Chapter 4.

### 3.3 Reduction of 2-nitro-benzonitrile **282**.

The reduction of 2-nitro-benzonitrile **282** by *S. cerevisiae* afforded 2-amino-benzamide **284** (major) and 2-amino-benzonitrile **283** (minor) (Fig 3-4). The incubation of 2-nitro-benzonitrile **282** with the isolated enzyme(s) recorded the presence of only 2-amino-benzamide **284**.



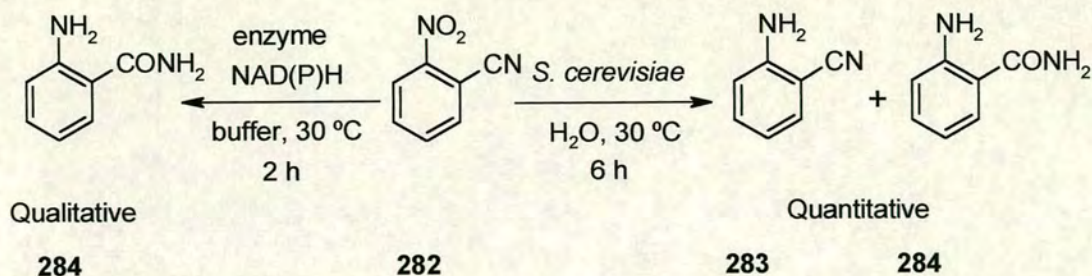
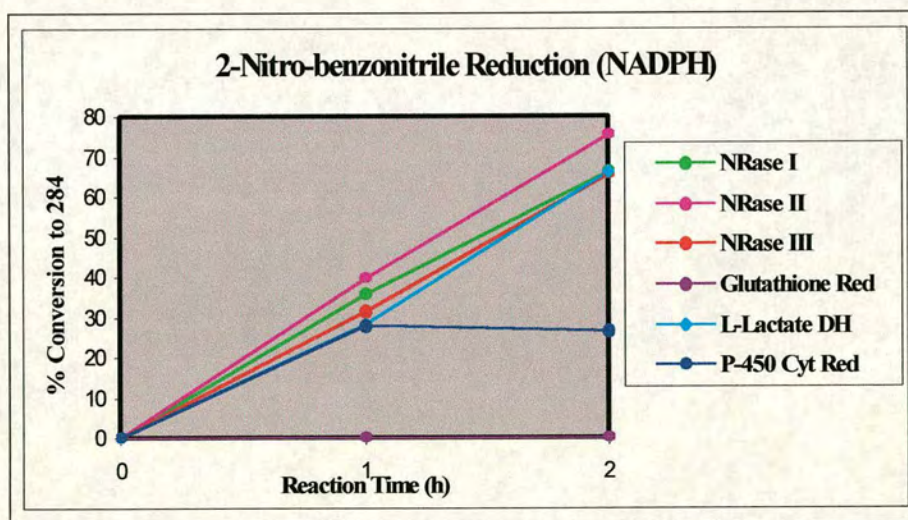


Fig 3-4 : Reduction of 2-nitro-benzonitrile **282**.

### 3.3.1 Reduction of 2-nitro-benzonitrile **282** using NADPH.

The substrate **282** was dissolved in the minimum amount of DMSO and added to a solution of the enzyme(s) and NADPH in tris buffer. The reduction of the substrate was monitored by GCMS with samples withdrawn at regular time intervals (1 h, 2h) from the reaction mixture. The results obtained are plotted in the form of Graph 3-3.



Graph 3-3 : Reduction of 2-nitro-benzonitrile **282** using isolated enzymes.

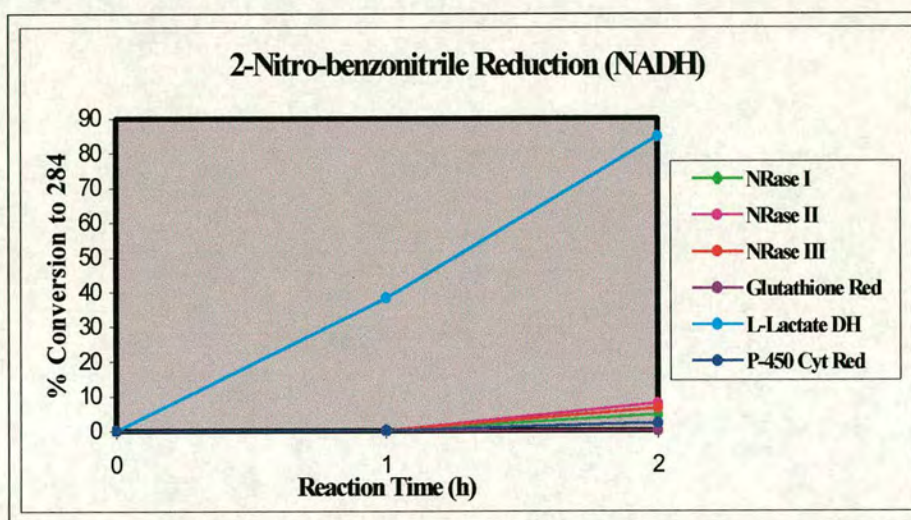
All 3 reductases (Nrases I, II and III) isolated from *S. cerevisiae* and L-lactate dehydrogenase reduced the nitro group over 2 h with a high percentage of 2-amino-benzamide **284** recorded. The maximum conversion to **284** levelled off for P-450



cytochrome reductase after 1 h and glutathione reductase displayed no reducing activity towards the nitro group.

### 3.3.2 Reduction of 2-nitro-benzonitrile **282** using NADH.

The substrate **282** was dissolved in the minimum amount of DMSO and added to a solution of the enzyme(s) and NADH in tris buffer. The reduction of the substrate was monitored by GCMS with samples withdrawn at regular time intervals (1 h, 2h) from the reaction mixture. The results obtained are plotted in the form of Graph 3-4.



Graph 3-4 : Reduction of 2-nitro-benzonitrile **282** using isolated enzymes.

A low percentage (less than 8 %) of the reduced product **283** was detected in all cases, with the exception of L-lactate dehydrogenase which recorded a high percentage of product **284** when NADH was used as a co-factor.

### 3.4 Conclusion.

All three purified reductases from *S. cerevisiae* were effective at reducing the nitro group in dinitrobenzene **320**. NADH or NADPH could be used as a co-factor, with



the latter case, the percentage of 4-nitro-aniline **321** produced increased over the course of the reaction. L-lactate dehydrogenase and P-450 cytochrome reductase displayed the ability to reduce the nitro group but were not as effective compared to the purified proteins, whereas glutathione reductase displayed no reducing activity.

All three purified enzymes reduced the nitro group with maximum efficiency in 2-nitro-benzonitrile **282** to afford 2-amino-benzamide **284** when NADPH was used. L-lactate dehydrogenase was the most effective at reduction when NADH was used and glutathione reductase failed to reduce the nitro group with either NADPH or NADH.

This may suggest that the three purified nitro-reductases from *S. cerevisiae* are the main enzymes responsible for the catalytic reduction of the nitro group in simple nitro aromatic compounds. Also, the individual enzymes may have different preferences for which cofactor is involved in the reduction process (NADH or NADPH). The other enzymes investigated are present in *S. cerevisiae* but act as background reductases which have some reducing activity but contribute almost nothing to the overall reduction process.

### **3.5 Nitro-reductases present in other microorganisms.**

To our knowledge, OYE II (NRase II) present in *S. cerevisiae* has not been previously reported as a nitro-reductase. The nitro reduction activity of OYE II was proven by over-expression of the gene encoded for OYE II and recombinant OYE II purification. The gene encoded for Lipoamide dehydrogenase (NRase I) from *S. cerevisiae* was also over-expressed, recombinant NRase I purified and shown as a nitro-reductase.<sup>94</sup> NRase I and Thioredoxin reductase (NRase III) present in other microorganisms have been reported to display nitro reduction activity. However, OYE II (NRase II) isolated from other microorganisms has not been reported to have nitro group reducing ability.



A lipoamide dehydrogenase from pig heart displayed nitro reductase activity in which the nitro group of 4-nitro-pyridine **324** and 4-nitro-pyridine *N*-oxide was reduced in the presence of NADH.<sup>145</sup> The major product from the enzymatic reduction of 4-nitro-pyridine **324** was characterised as *NN*-bis(pyridinyl)hydroxylamine **326**, which was formed presumably through a hydroxyaminopyridine intermediate **325** (Fig 3-5).

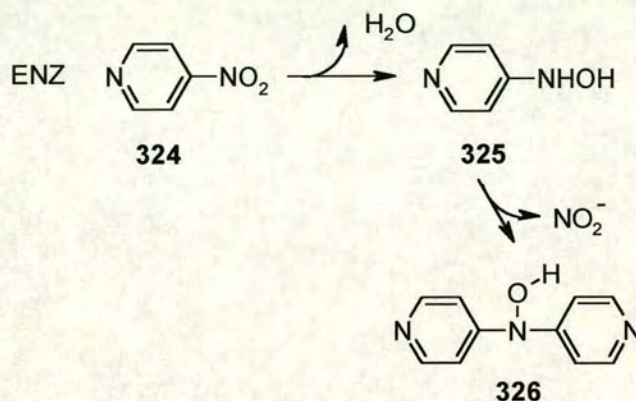


Fig 3-5 : Proposed mechanism for the reduction of 4-nitro-pyridine **324**.

A recent paper reported on the reducing ability of a thioredoxin reductase from *Mycobacterium tuberculosis*, which was expressed in *Escherichia coli* on dinitrobenzene substrates.<sup>146</sup> The reduction of 2,4-dinitro-chloro-benzene **327a** by thioredoxin reductase yielded 4-chloro-3-nitro-aniline **328a** (major) in which the *para* nitro group was reduced and 2-chloro-5-nitro-aniline **329a** (minor). In contrast, the reduction of 2,4-dinitro-toluene **327b** afforded only 4-amino-2-nitro-toluene **328b** in which the *para* nitro group was reduced (Fig 3-6).

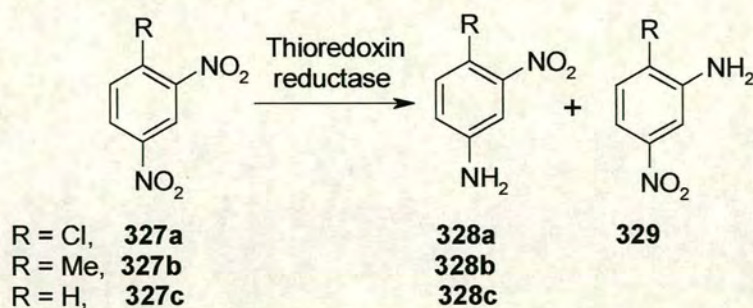


Fig 3-6 : Thioredoxin reductase reduction in dinitrobenzene substrates.



The reduction of one nitro group in 1,3-dinitrobenzene **327c** afforded the expected 3-nitro-aniline product **328c**, but mono-nitrobenzenes were not reduced by the thioredoxin reductase.

### 3.6 Regioselective reduction.

The approach of using purified nitro-reductases on simple nitro aromatic compounds was extended further to determine whether any selectivity was observed with potential substrates while using these reductases. One possible substrate was 2,4-dinitro-anisole **330** which was reduced by *S. cerevisiae* to afford **331** and **332** in yields of 80 % and 15 % respectively, therefore representing a regioselective reduction in favour of the *ortho*-amino product **331** (Fig 3-7).

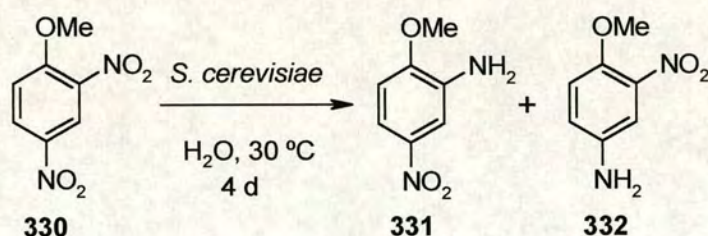


Fig 3-7 : *S. cerevisiae* catalysed reduction of 2,4-dinitro-anisole **330**.

The progress of the reduction of 2,4-dinitro-anisole **330** was monitored by GCMS with samples withdrawn at day 2 from the reaction mixture. Three peaks were recorded by the GCMS which corresponded to the starting material **330** and the two reduced regioisomeric products **331**, **332** (Fig 3-8).



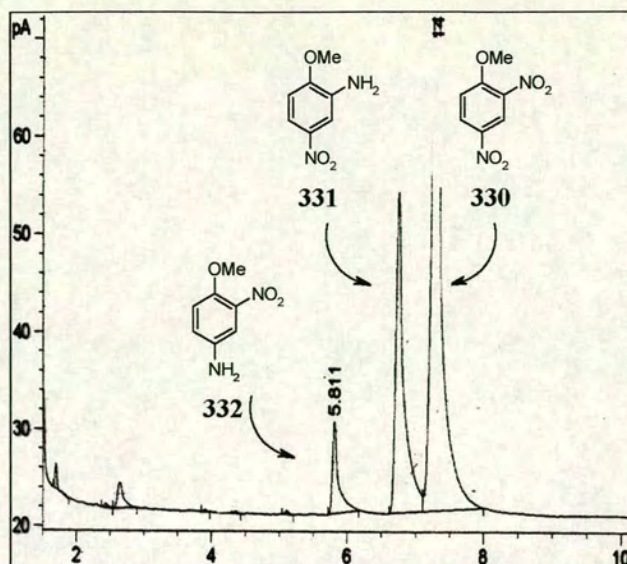


Fig 3-8 : *S. cerevisiae* catalysed reduction of 2,4-dinitro-anisole **330** after 2d.

It was proposed that the nitro-reductases from *S. cerevisiae* might produce a different ratio of both regioisomers. A promising result was obtained when the substrate **330** was incubated with NRase II and the progress of the reduction was monitored by GCMS. Only the peaks corresponding to the *ortho*-amino product **331** and the starting material **330** were recorded after 2 d (Fig 3-9).

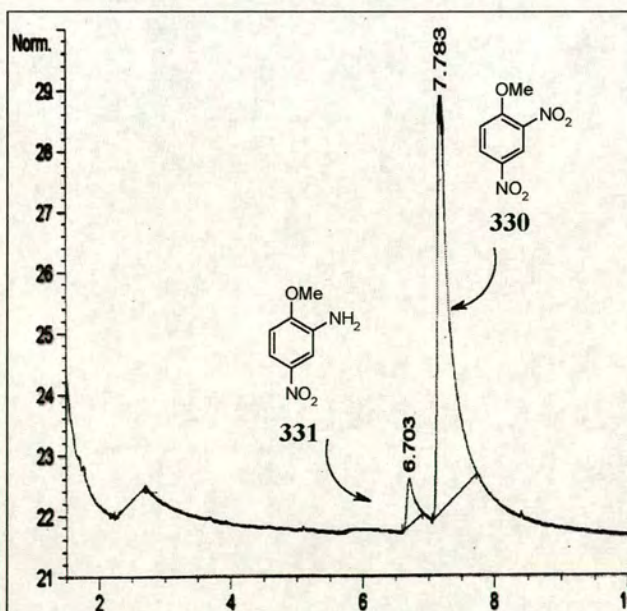


Fig 3-9 : NRase II catalysed reduction of 2,4-dinitro-anisole **330** after 2d.



This suggested that perhaps the individual nitro-reductases or NRase II was more selective than the whole-cell system itself. These are preliminary studies and further studies have to be carried out on simple nitro aromatic compounds with these purified nitro-reductases in order to determine the selectivities of these isolated systems.



## 4. Results & Discussion : Biotransformation Mechanism.

This chapter describes work carried out to elucidate the mechanism of nitro reduction mediated by *S. cerevisiae*. A thorough investigation into the reduction of 3-nitro-phthalonitrile has been conducted. Further insights into the mechanism and the intermediate(s) involved have been achieved by GCMS, HPLC, LCMS and isotopic labelling studies. The possibility of tautomerisation of the proposed benzo-isoxazolidine intermediate has also been discussed.

### 4.1 Mechanistic studies involving 2-nitrobenzonitrile **282**.

Initial studies were carried out on the reduction of 2-nitro-benzonitrile **282** by *S. cerevisiae* with 2-amino-benzamide **284** obtained as the major product, where the nitro group in **282** was reduced and the cyano group was converted into the amide functionality (Fig 4-1).

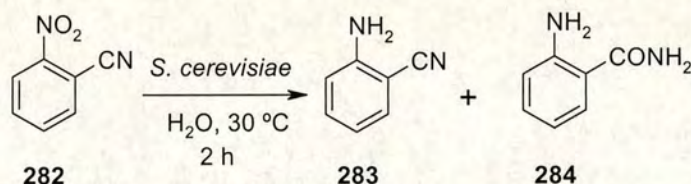


Fig 4-1 : *S. cerevisiae* catalysed reduction of 2-nitro-benzonitrile **282**.

Previous work carried out by Davey *et al.* led to a proposed mechanism for the reduction process (Fig 4-2).<sup>2</sup> It was believed that the initial step involved the reduction of the nitro group in **282** to afford the hydroxylamine **333**, which underwent intramolecular attack by the hydroxy group to yield the isoxazolidine derivative **334**. A further 2-electron reduction of the benzo-isoxazolidine **334** resulted in the cleavage of the N-O bond to afford 2-amino-benzamide **284**.



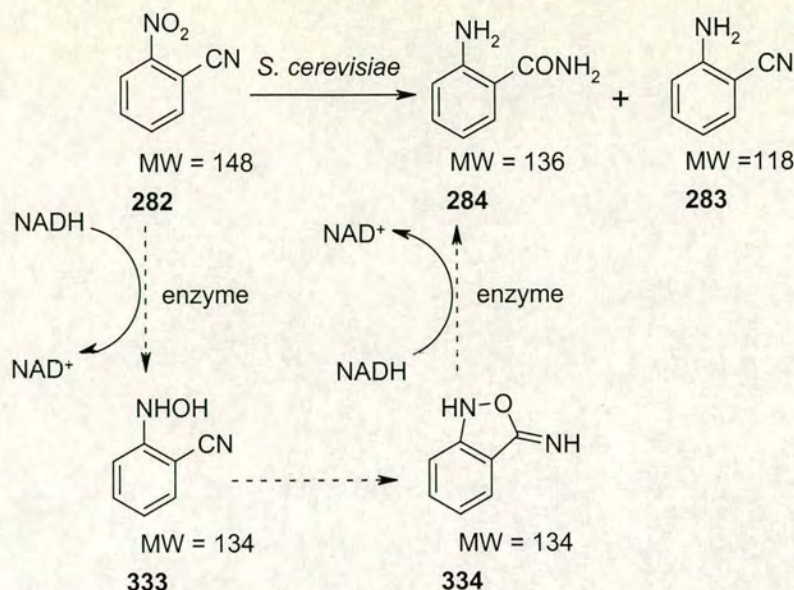


Fig 4-2 : Proposed mechanism for the reduction of 2-nitro-benzonitrile **282**.

The progress of the reduction of the substrate **282** was monitored by GCMS with samples withdrawn at fixed time intervals from the reaction mixture. A peak was detected which appeared and then disappeared suggesting that an intermediate was involved in the process. A molecular ion with a molecular weight of 134 Da was obtained when the peak was scanned by the mass spectrometer. The intermediates **333** and **334** both have a molecular weight of 134 (Fig 4-3), providing supporting evidence for the proposed mechanism outlined in Fig 4-2.

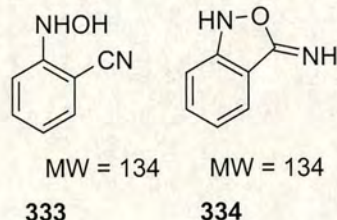


Fig 4-3 : Proposed intermediates in the reduction of 2-nitro-benzonitrile **282**.

*Ortho*-dicyanobenzene **335** was then incubated with *S. cerevisiae* to determine whether cyano groups on the substrate might be converted to the amide without the presence of a nitro group. A successful conversion of this compound would suggest that the transformation of the CN group to the amide might be carried out by enzymes present in *S. cerevisiae* that are different to those that are specific for nitro



reduction. The biotransformation was monitored by GCMS with the starting material **335** recovered in quantitative yield over a few days (Fig 4-4).

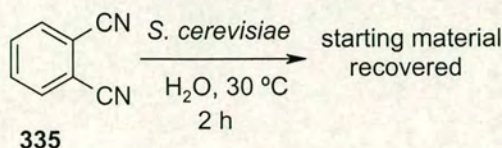


Fig 4-4 : Incubation of *ortho*-dicyanobenzene with *S. cerevisiae*.

Therefore, it was found that the relative positions of the nitro and cyano groups were important. This was confirmed by the incubation of 3- and 4-nitro-benzonitrile **285**, **287** with *S. cerevisiae* which afforded only the corresponding amino-benzonitriles **286**, **288** with the *meta*- and *para*- substituted cyano groups remaining intact (Fig 4-5). Hence, a nitro group substituted *ortho* to the cyano group on the aromatic ring is required for the conversion of this cyano group to take place.

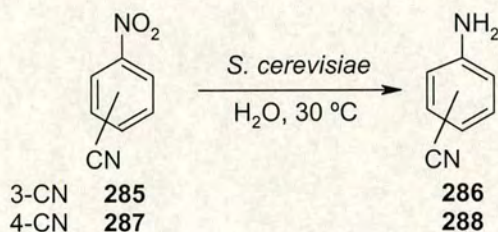


Fig 4-5 : *S. cerevisiae* catalysed reduction of 3- and 4-nitro-benzonitrile.

In view of the fact that the level of intermediate obtained during the reduction of 2-nitro-benzonitrile **282** was not sufficiently high to carry out further mechanistic studies, it was decided to examine, the related substrate 3-nitro-phthalonitrile **238** in more depth.



## 4.2 Mechanistic studies involving 3-nitro-phthalonitrile **238**.

*S. cerevisiae* catalysed reduction of 3-nitro-phthalonitrile **238** afforded two products, 2-amino-6-cyano-benzamide **289** (major) and 2-amino-6-cyano-benzoic acid **290** (minor) in yields of 50 % and 10 % respectively (Fig 4-6).

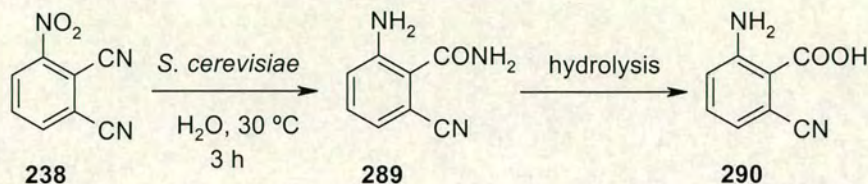


Fig 4-6 : *S. cerevisiae* catalysed reduction of 3-nitro-phthalonitrile **238**.

A similar mechanism (Fig 4-2) has been proposed for this reduction process<sup>69</sup>, in which the first step is the initial reduction of the nitro group in **238** to afford **336** followed by intramolecular attack to yield the benzo-isoxazolidine derivative **337**. A further 2-electron reduction resulted in the cleavage of the N-O bond to afford 2-amino-6-cyano-benzamide **289** (Fig 4-7). The formation of the acid **290** via the hydrolysis of the amide **289** during the incubation with *S. cerevisiae* has been discussed in Chapter 2B.

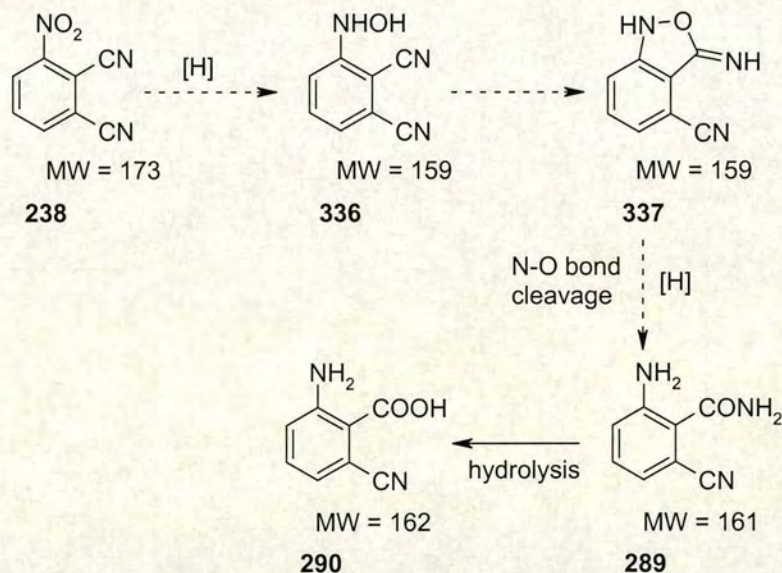


Fig 4-7 : Proposed mechanism for the reduction of 3-nitro-phthalonitrile **238**.



3-Nitro-phthalonitrile **238** was then incubated with *S. cerevisiae* which was suspended in labelled  $^{18}\text{O}$ -water, to determine whether the oxygen incorporated in the amide product **289** was obtained either from the medium (water) or from the nitro group of the starting material **238** (Fig 4-8). The small scale biotransformation was monitored by GCMS with a sample removed at day 2 from the reaction mixture.

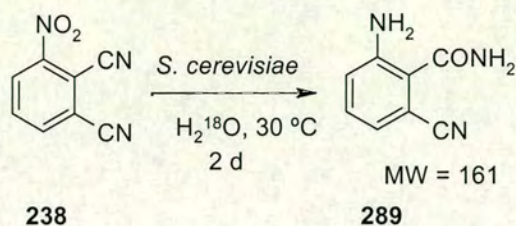


Fig 4-8 : *S. cerevisiae* catalysed reduction of 3-nitro-phthalonitrile **238**.

A peak corresponding to the amide product **289** was detected, which recorded a mass ion of 161 Da, therefore the labelled  $^{18}\text{O}$  from water (medium) was not incorporated into the amide **289**, since if this was the case, a mass ion of 163 Da for the amide product **289** would be recorded. This suggested that bicyclic type of intermediate(s) are involved in the reduction process in order for the oxygen of the nitro group in the starting material **238** to be incorporated into the amide functionality of the final product **289**.

This proposed mechanism is supported by Navarro-Ocaña who reported on the formation of substituted isoxazoles **122** from (*Z*)-aryl- and (*Z*)-alkyl-2-phenyl-3-nitro-propenenitriles **121** (Fig 4-9).<sup>6</sup>



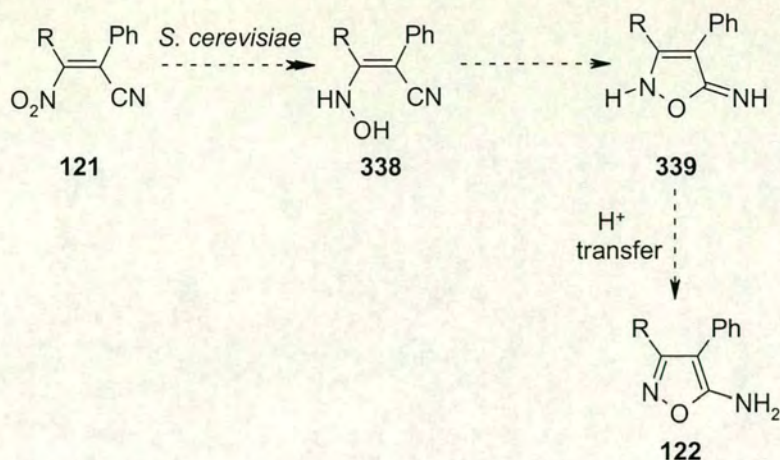


Fig 4-9 : *S. cerevisiae* catalysed reduction of nitroalkenes.

The initial step was believed to be the reduction of the nitro group in **121** to afford the hydroxylamine derivative **338** followed by intramolecular attack to yield the heterocyclic structure **339** followed by proton transfer to form the isoxazole derivative(s) **122**. The relative stability of the ring systems of the substituted amino-isoxazoles **122** which were formed is believed to account for the fact that further N-O bond cleavage did not occur. The substrates studied in this chapter apparently do not form such a stable intermediate and hence, N-O bond cleavage of **337** occurs to afford the amino-amide product **289**.

#### 4.2.1 GCMS studies.

The progress of the reduction of 3-nitro-phthalonitrile **238** was monitored by GCMS with samples withdrawn at fixed time intervals from the reaction mixture. A peak at 13.4 min was observed to appear and disappear during the course of the biotransformation, suggesting that this might correspond to intermediate(s) involved in the reduction pathway (Fig 4-10 i-v).



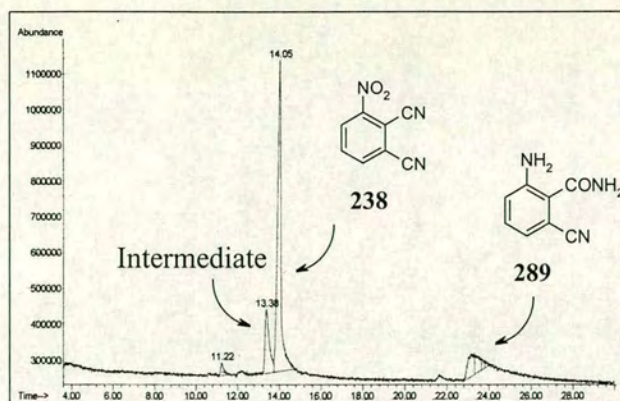


Fig 4-10i : Reaction time = 5 min.

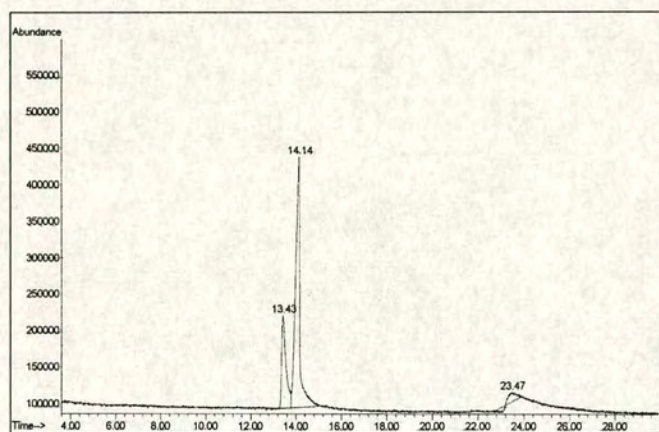


Fig 4-10ii : Reaction time = 12 min.

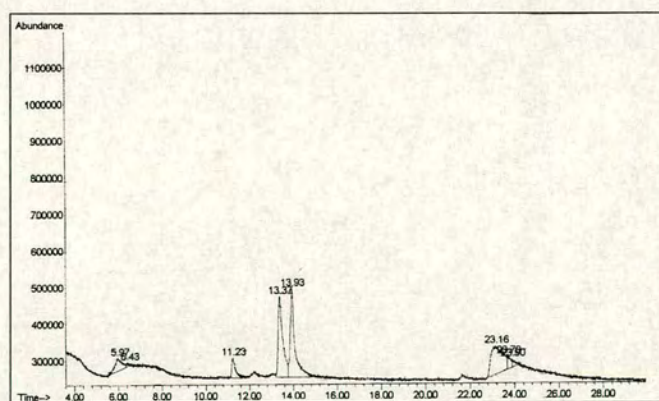


Fig 4-10iii : Reaction time = 20 min.



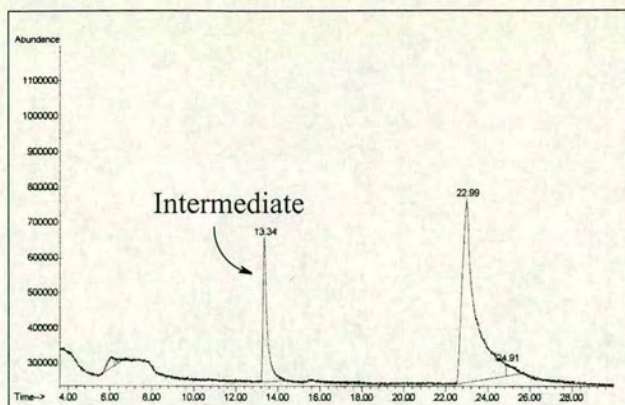


Fig 4-10iv : Reaction time = 30 min.

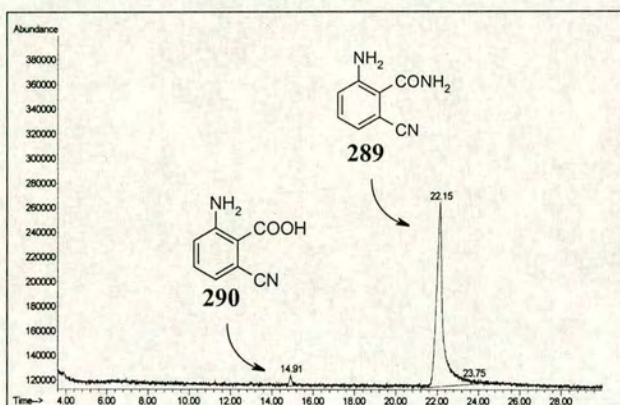


Fig 4-10v : Reaction time = 4 h.

Mass spectral analysis of the peak corresponding to a retention time of 13.4 min indicated a molecular ion with a molecular weight of 161 Da. The mass spectrum for the intermediate had a similar fragmentation pattern to that of the amide product **289** (Fig 4-11 i-ii).



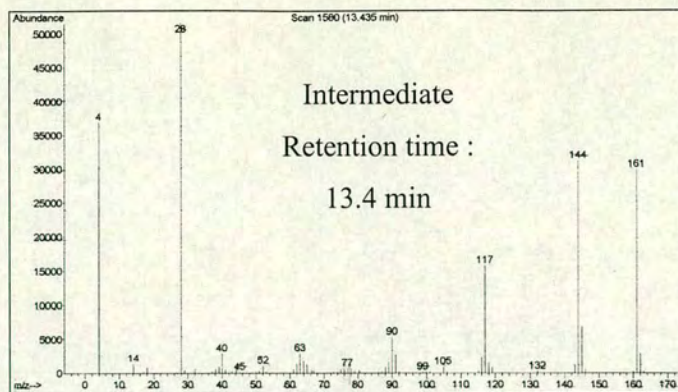


Fig 4-11i : Mass spectrum of the intermediate peak (13.4 min).

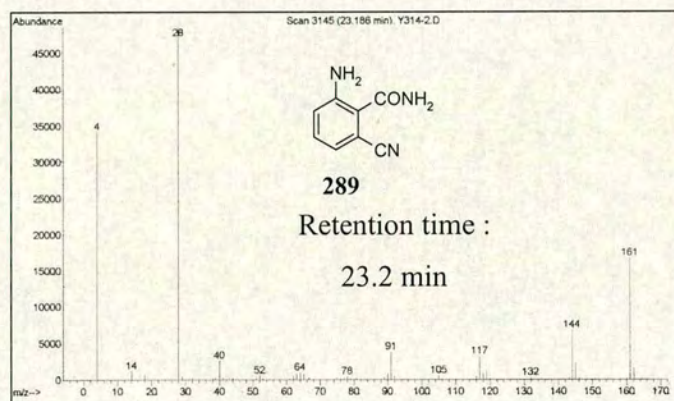


Fig 4-11ii : Mass spectrum of the amide peak (23.2 min).

It was evident from the mass spectrum obtained for the intermediate that a molecular ion of 161 Da was present even though both intermediates **336** and **337** in the proposed mechanism have a molecular weight of 159 (Fig 4-12).

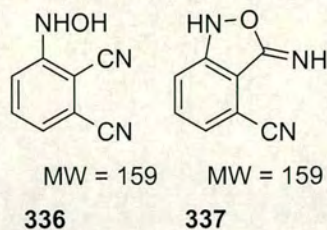


Fig 4-12 : Proposed intermediates in the reduction of 3-nitro-phthalonitrile **238**.



The reason for the differences in molecular weights may be explained by the procedure in which the samples withdrawn from the reaction mixture were analysed. A high oven temperature was required to detect the intermediate in the reduction of 3-nitro-phthalonitrile **238**. Due to the high temperature, the intermediate may be converting to the amide **289** as it enters the mass spectrometer whose mass spectrum (mass ion of 161 Da) is recorded corresponding to the retention time (13.4 min) of the peak for the intermediate.

The peak corresponding to a retention time of 13.4 min was not the only intermediate peak recorded, other peak(s) which were low in concentration were also detected.

#### **4.2.2 Further studies into the intermediate including attempts to isolate.**

The major intermediate in the reduction of 3-nitro-phthalonitrile **238** proved very difficult to isolate. Purification methods such as flash-column and reverse-phase chromatography with different eluting conditions were attempted but failed to yield the pure material. The biotransformation was stopped at 30 min, when the intermediate concentration was at a maximum, although various work-ups were attempted all methods failed to confirm the structure of the intermediate.

#### **4.2.3 HPLC studies.**

Studies using an analytical HPLC system were carried out and with the intention of optimisation of conditions followed by preparative HPLC to isolate the intermediate. Close monitoring of the biotransformation by analytical HPLC using optimised conditions (40 % MeCN, 60 % H<sub>2</sub>O, (0.1 % TFA), 254 nm, 1 mL/min) clearly showed the presence of an intermediate peak. The progress of the reduction of 3-nitro-phthalonitrile **238** as monitored by HPLC is shown below (Fig 4-13 i-v).



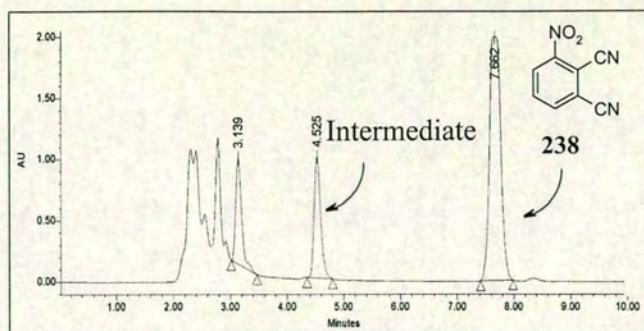


Fig 4-13i : Reaction time = 2 min.

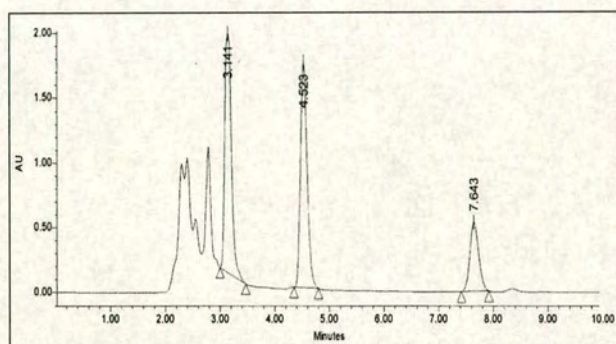


Fig 4-13ii : Reaction time = 12 min.

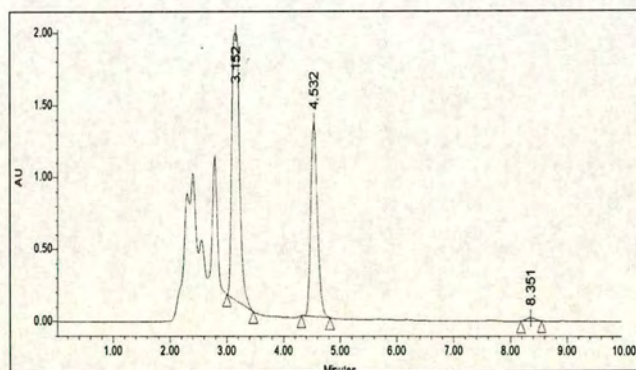


Fig 4-13iii : Reaction time = 25 min.



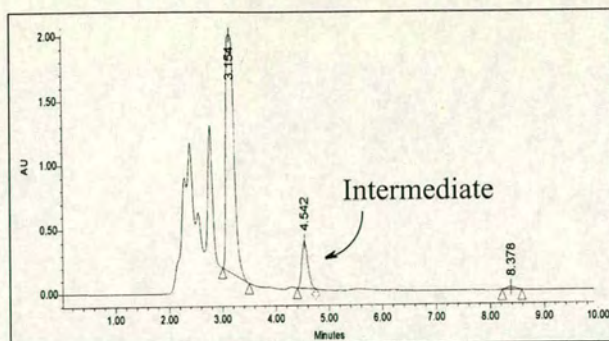


Fig 4-13iv : Reaction time = 45 min.

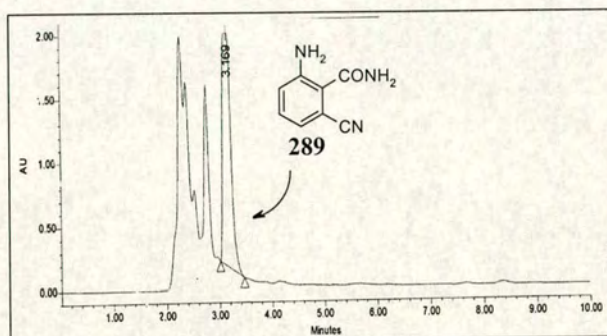


Fig 4-13v : Reaction time = 1 h.

A range of mobile phase conditions was employed to give the best separation between the intermediate and the other peaks present for preparative work (10 % MeCN, 90 % H<sub>2</sub>O, 254 nm, 4.7 mL/min). Fractions corresponding to the intermediate were collected and after evaporation of the solvent, were analysed by GCMS, which showed either mixtures of both the intermediate and the amide product **289** or no intermediate at all.



## 4.2.3 LCMS studies.

In contrast to GCMS, electrospray LCMS is a milder technique and was carried out in an attempt to obtain molecular weight characterisation of the intermediate in the reduction of 3-nitro-phthalonitrile **238**. The progress of the reduction was monitored by LCMS with samples withdrawn at fixed time intervals from the reaction mixture using the optimised conditions (Fig 4-14).

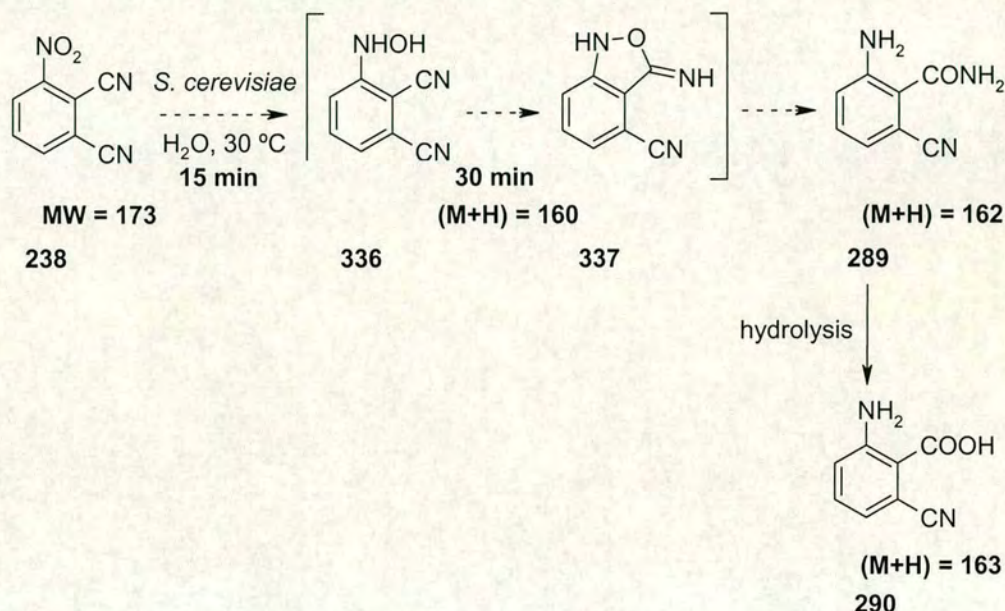


Fig 4-14 : *S. cerevisiae* catalysed reduction of 3-nitro-phthalonitrile **238** monitored by LCMS.

A peak corresponding to a retention time of 6.10 min was observed to appear and then disappear suggesting that this may be the intermediate that was also recorded by GCMS and HPLC. The progress of the reduction of 3-nitro-phthalonitrile **238** as monitored by LCMS is shown in Fig 4-15 i-v.



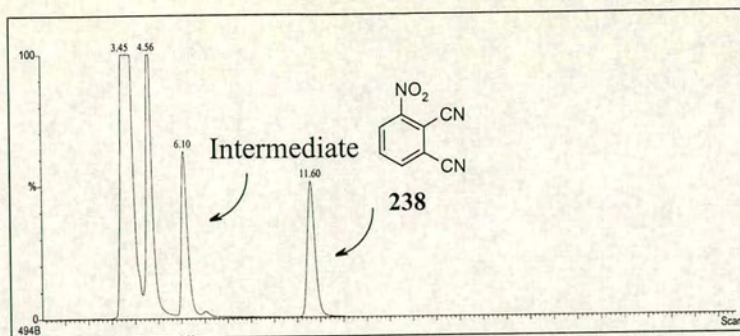


Fig 4-15i : Reaction time = 50 min.

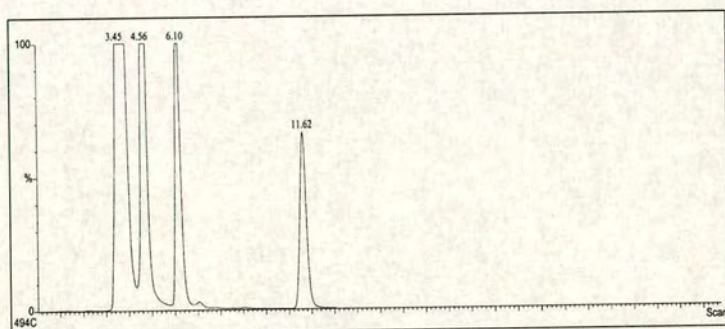


Fig 4-15ii : Reaction time = 15 min.

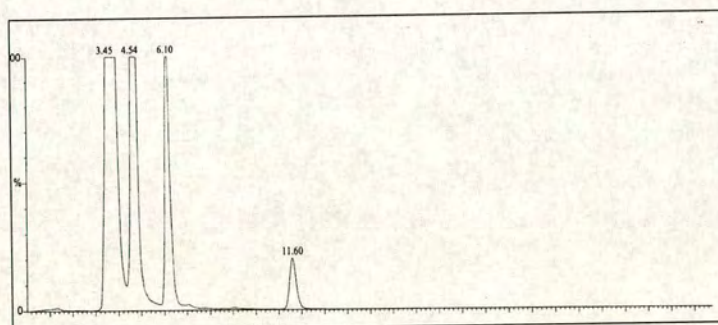


Fig 4-15iii : Reaction time = 25 min.



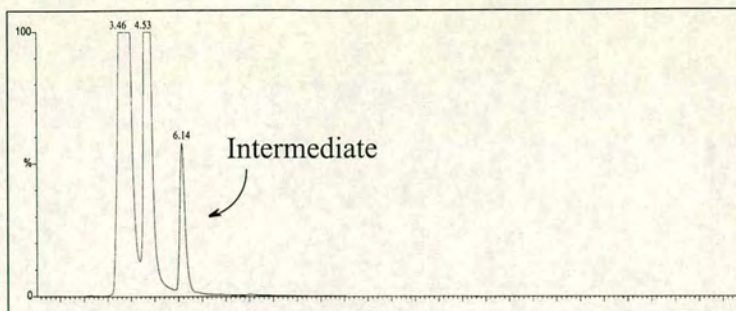


Fig 4-15iv : Reaction time = 40 min.

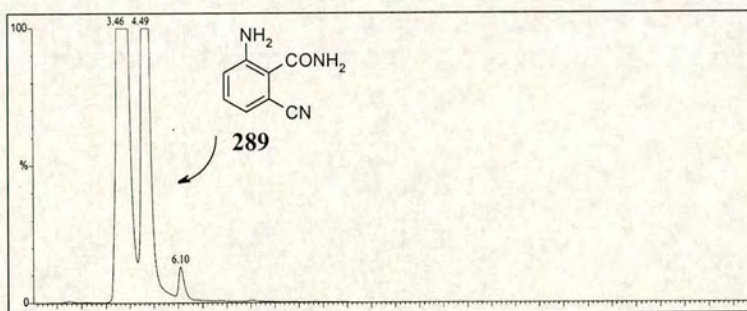


Fig 4-15v : Reaction time = 55 min.

The peaks corresponding to the amide **289** and acid **290** products afforded the expected molecular ion (M+H) with molecular weights of 162 Da and 163 Da respectively. The mass spectrum of the peak corresponding to the intermediate indicated a molecular ion (M+H) of 160, which further reinforced the proposal that the intermediate(s) are likely to be the hydroxylamine **336** or the benzo-isoxazolidine **337** derivative in this reduction process (Fig 4-16). Both these intermediates have a molecular weight of 159 Da and when subjected to LCMS conditions would expect to give rise to two peaks with different retention times having the same mass ion of 160 Da.



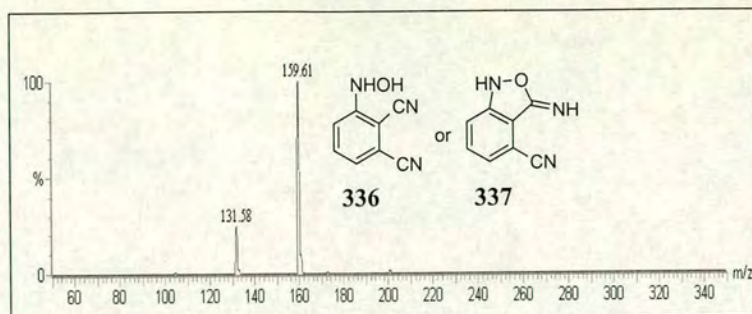


Fig 4-16 : Mass spectrum of the intermediate peak (6.1 min).

### 4.3 Dicyanonitroarene studies.

Further insight into the mechanism in terms of what intermediate(s) were involved was gained by investigating the reduction of other dicyanonitroarene substrates. As discussed in Chapter 2B, the six possible isomers in which two cyano groups are substituted on the aromatic ring along with the nitro group were incubated with *S. cerevisiae*. Those substrates in which a cyano group was substituted *ortho* to the nitro group on the aromatic ring underwent selective conversion to the corresponding amide moiety. The reduction of the dicyanonitroarene substrates **221**, **225**, **230**, **233**, **294** were monitored by GCMS, HPLC and LCMS. An intermediate peak was recorded in substrates **225**, **230**, **233** all of which have a cyano group substituted *ortho* to the nitro group on the aromatic ring (Fig 4-17).

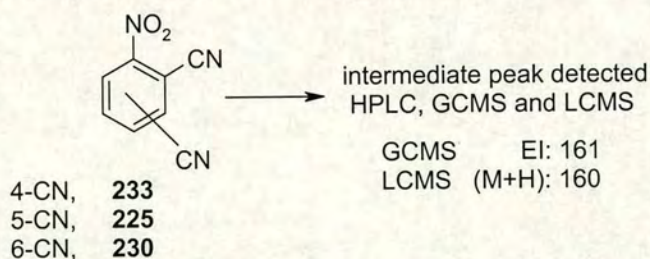


Fig 4-17 : Intermediate recorded in dicyanonitroarene substrates.

The mass spectrum (GCMS) of the peak corresponding to the intermediate(s) had the same fragmentation pattern as that of their corresponding amide product(s). Whereas,



LCMS studies clearly indicated the presence of an intermediate(s) peak with a molecular ion (M+H) of 160 Da.

No intermediate was detected by HPLC, GCMS or LCMS in substrates **221**, **294** all of which have no cyano group substituted *ortho* to the nitro group on the aromatic ring (Fig 4-18).

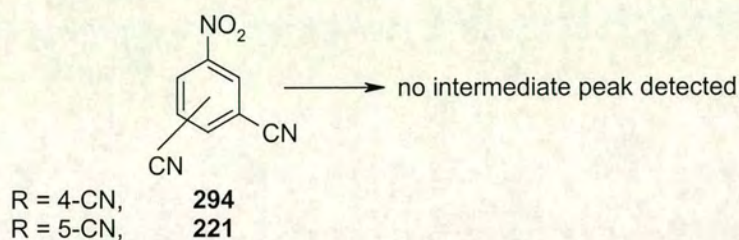


Fig 4-18 : No intermediate recorded in theses dicyanonitroarene substrates.

Therefore, the intermediate detected in the reduction of dicyanonitroarenes was similar in structure to the benzo-isoxazolidine derivative **337** and not to the hydroxylamine compound **336**. Since, an intermediate was only detected in those substrates which have a cyano group substituted *ortho* to the nitro group on the aromatic ring implying that both the nitro group and *ortho* cyano group play a significant part in the reduction process and may be transformed into the benzo-isoxazolidine derivative **337** (Fig 4-18) which was the likely intermediate (Fig 4-19).

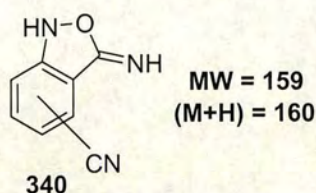


Fig 4-19 : Likely intermediate in the reduction of dicyanonitroarenes.



#### 4.4 Isotopic labelling studies.

$^{13}\text{C}$ Carbon isotopic labelling studies of substrates **342**, **343** were carried out to gain further evidence that the benzo-isoxazolidine structure **340** was the likely intermediate which was detected by GCMS, HPLC and LCMS in the reduction of (di)cyanonitroarene substrates catalysed by *S. cerevisiae*.

##### 4.4.1 $^{13}\text{C}$ -2-nitro-benzonitrile **342** studies.

Initial studies were carried out on labelled  $^{13}\text{C}$ -2-nitro-benzonitrile **342** which was synthesised by a nucleophilic displacement type of reaction.<sup>147</sup> Displacement of chloride from *ortho*-chloro-nitrobenzene **341** by  $^{13}\text{C}$ -labelled copper cyanide afforded the labelled substrate **342** in a moderate yield of 55 % (Fig 4-20).

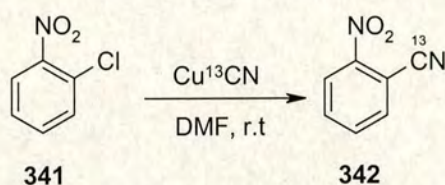


Fig 4-20 : Synthesis of labelled  $^{13}\text{C}$ -2-nitro-benzonitrile **342**.

The conditions for the biotransformation were optimised using 2-nitro-benzonitrile **282** as the substrate for the *S. cerevisiae* catalysed reduction to achieve the maximum concentration of the intermediate. The biotransformation was repeated using the labelled substrate **342** with the optimised conditions and the reaction was stopped at different time intervals. Subsequent work-up was carried out to afford the crude product which was analysed by GCMS and  $^{13}\text{C}$  NMR. Unfortunately, the intermediate was barely detectable by GCMS, which made it very difficult for the labelled carbon to be detected by  $^{13}\text{C}$  NMR, which requires a high concentration of the intermediate.



#### 4.4.2 2-<sup>13</sup>C-3-nitro-phthalonitrile **343** studies.

The progress of the reduction of 3-nitro-phthalonitrile **238** was monitored by GCMS, HPLC and LCMS which indicated a very high concentration of the intermediate present at 30 min reaction time. Therefore, the synthesis of labelled 2-<sup>13</sup>C-3-nitro-phthalonitrile **343** and subsequent reduction of **343** by *S. cerevisiae* was carried out. The progress of the reduction of **343** would be followed by <sup>13</sup>C NMR by stopping the reaction at fixed time intervals followed by work-up and analysis of the crude mixture by NMR (Fig 4-21).

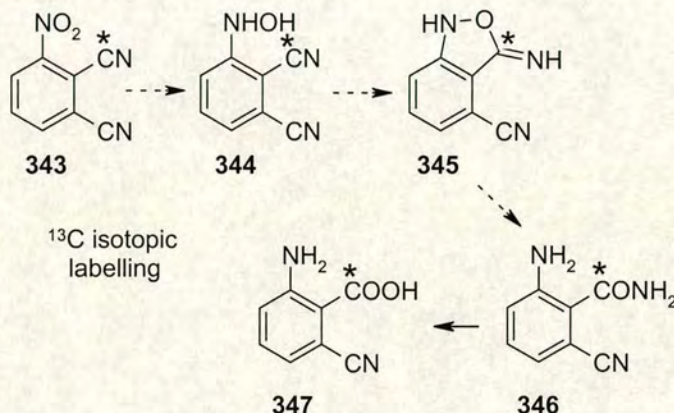


Fig 4-21 : *S. cerevisiae* catalysed reduction of 2-<sup>13</sup>C-3-nitro-phthalonitrile **343**.

The ppm value obtained in the <sup>13</sup>C NMR would give some indication as to what the nature and surrounding environment of the labelled carbon on the intermediate. The labelled carbon of the hydroxylamine intermediate **344** would be similar in value to the starting material **343**, in the region of about 115 ppm. Whereas the labelled carbon of the bicyclic intermediate **345** would be similar to the amide product **346**, a high value in the region of about 160 ppm.

The synthesis of labelled 3-nitro-phthalonitrile **343** involved a 4-step process which was similar to the synthesis of dicyanonitroarenes as described in Chapter 2A (Fig 2-1). 2-Bromo- or 2-chloro-3-nitro-benzoic acid **348** was converted to the corresponding acid chloride **349** by thionyl chloride, which was then converted to the amide **350** using concentrated aqueous ammonia solution. Further dehydration



resulted in the formation of the corresponding nitrile(s) **351** in moderate yields (Fig 4-22).

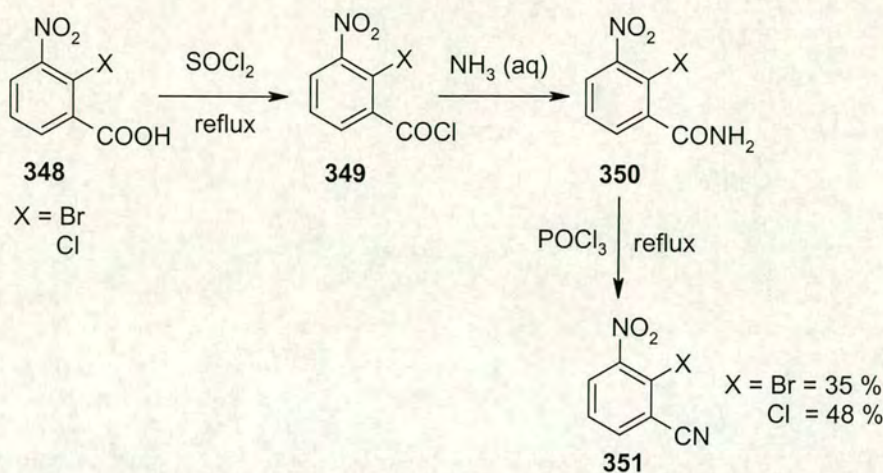


Fig 4-22 : Synthesis of 2-bromo or chloro-3-nitro-benzonitrile **351**.

The final step involved the displacement of the halogen in **351** with  $^{13}\text{C}$ -copper cyanide in dimethylacetamide under a dry nitrogen atmosphere to afford the labelled substrate **343** (Fig 4-23).

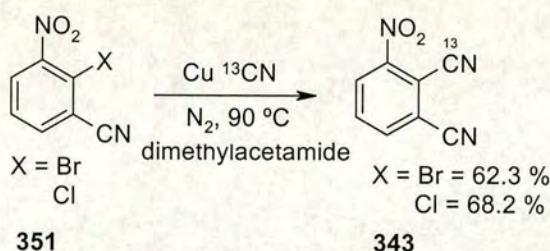


Fig 4-23 : Synthesis of 2- $^{13}\text{C}$ -nitro-phthalonitrile **343**.

The conditions for the biotransformation were optimised using 3-nitro-phthalonitrile **238** as the substrate for *S. cerevisiae* catalysed reduction to achieve the maximum concentration of the intermediate. The biotransformation was then repeated using the labelled substrate **343** and stopped at fixed time intervals. The crude product(s) obtained was analysed using  $^{13}\text{C}$  NMR to determine the ppm value of the labelled carbon. Close monitoring by  $^{13}\text{C}$  NMR clearly showed the presence of the labelled carbon of the starting material **343**, intermediate, amide **346** and acid **347** compounds (Fig 4-24 i-iii).



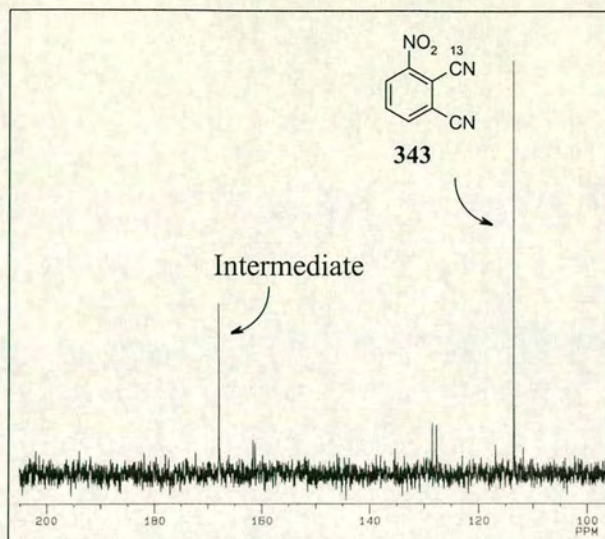


Fig 4-24i : Reaction time = 15 min.

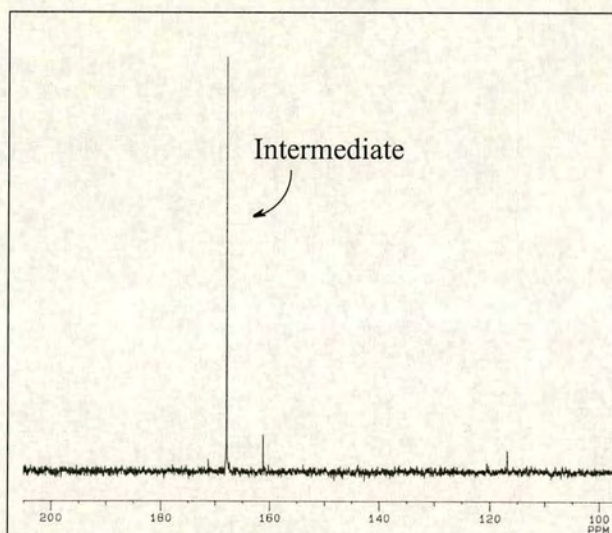


Fig 4-24ii : Reaction time = 35 min.



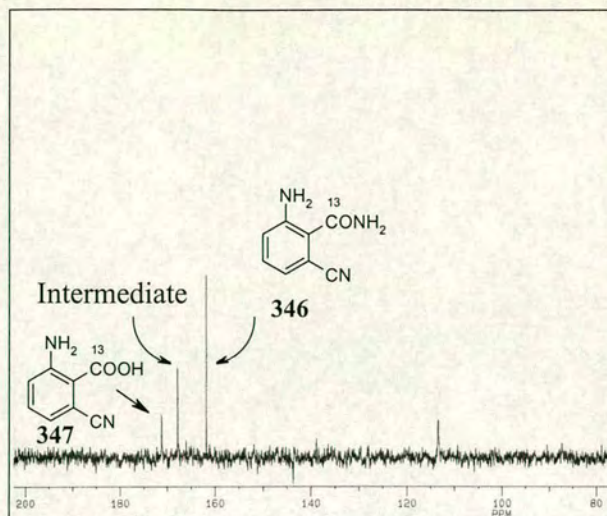


Fig 4-24iii : Reaction time = 50 min.

The  $^{13}\text{C}$  ppm values obtained from the reactions involving the labelled substrate **343** are tabulated below along with the literature values (Table 4-1).

	Starting material	Amide	Acid	Intermediate
$\delta^{13}\text{C}$	113.4 ppm	162.1 ppm	171.2 ppm	167.9 ppm
Literature	113.1 ppm	167.8 ppm	-----	-----

Table 4-1:  $^{13}\text{C}$  NMR chemical shifts of compounds in the reduction of 3-nitro-phthalonitrile **343**.

The  $^{13}\text{C}$  NMR results clearly showed that the labelled carbon corresponding to the intermediate had a value of 167.9 ppm which was similar to the value of the amide product **346**. Therefore, the proposed bicyclic compound **345** might be the likely intermediate which was detected in the reduction process mediated by *S. cerevisiae* (Fig 4-25).



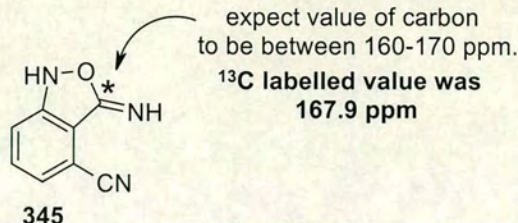


Fig 4-25 : Likely intermediate in the reduction of 3-nitro-phthalonitrile **238/343**.

The <sup>13</sup>C shift was observed for the carbon of interest in compounds similar to the benzo-isoxazolidine derivative **345**. 3-Amino-isoindol-1-one **352** was reported to have a value of 164.5 ppm for the carbon of interest (Fig 4-26).<sup>148</sup>

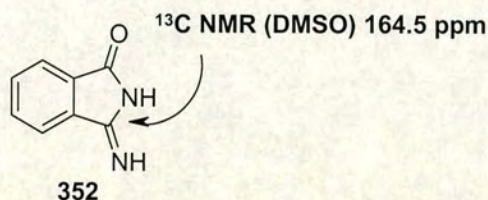


Fig 4-26 : Carbon value for 3-amino-isoindol-1-one **352**.

As mentioned previously, Navarro-Ocaña reported on the formation of substituted isoxazoles **122**, which were similar in structure to the proposed intermediate (Fig 4-27).<sup>6</sup> Spectroscopic data was given for **122** (R = Ph), where the carbon of interest had a value of 166.4 ppm.

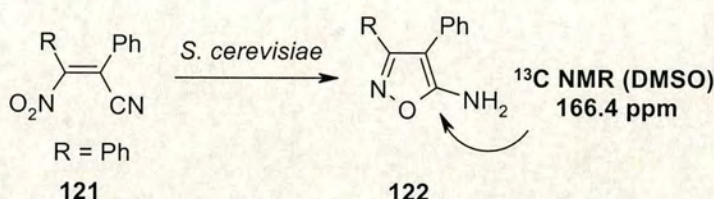


Fig 4-27 : *S. cerevisiae* catalysed reduction of nitroalkenes.

Both the compounds **122**, **352** have values corresponding to the carbon of interest in the range of about 165 ppm, which was similar to that of the intermediate **345** (167.9 ppm).



#### 4.5 Conclusion.

Further insight into the mechanism employed by *S. cerevisiae* in the nitro group reduction of (di)cyanonitroarenes has been gained. The intermediate (major) detected proved very difficult to isolate but studies have shown and further confirmed what intermediate(s) are likely to be involved in the mechanism. It was evident from the GCMS and HPLC data obtained that an intermediate peak was only detected in the biotransformations of substrates that bear a cyano group substituted *ortho* to the nitro group on the aromatic ring. Therefore, it is postulated that this cyano group plays a significant role in the reduction pathway employed by *S. cerevisiae*.

The introduction of isotopically  $^{13}\text{C}$ -labelled 3-nitro-phthalonitrile studies showed that the labelled carbon of the intermediate had a value of 167.9 ppm. This value was similar to the amide product and similar compounds that have been reported in the literature. Hence, the intermediate detected in the catalysed reduction of dicyanonitroarenes is likely to be the benzo-isoxazolidine compound **353** (Fig 4-28).

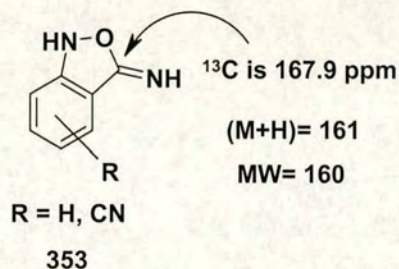


Fig 4-28 : Likely intermediate in the catalysed reduction of (di)cyanonitroarenes.

It would be interesting to see what the end results would be when the labelled substrates **342** and **343** are incubated with nitro-reductases which have been isolated from *S. cerevisiae* with the progress of reduction monitored by GCMS.



#### 4.6 Tautomerisation.

The proposed cyclic intermediate **353** in the reduction of (di)cyanonitroarenes could exist in two forms **353** and **354** which are tautomers of each other (Fig 4-29). The next stage attempted to elucidate which tautomer was the one that was present during the biotransformations of the dicyanonitroarenes.

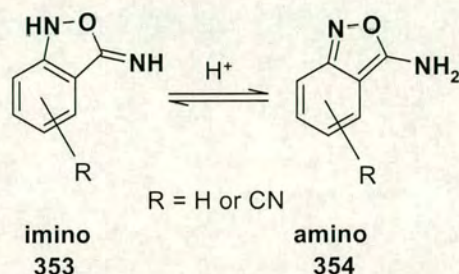


Fig 4-29 : Tautomers of the benzo-isoxazolidine intermediate.

A review on anthranils has discussed the catalytic reduction of 2-nitro-benzonitrile **282** where 3-amino-anthranil **355** was detected as an intermediate *en route* to 2-amino-benzamide **284** (Fig 4-30).<sup>149</sup> The original authors have reported on the reduction of 2-nitro-benzonitrile **282** over a platinum catalyst in which 3-amino-anthranil **355** was detected as an intermediate to **284**.<sup>150</sup>

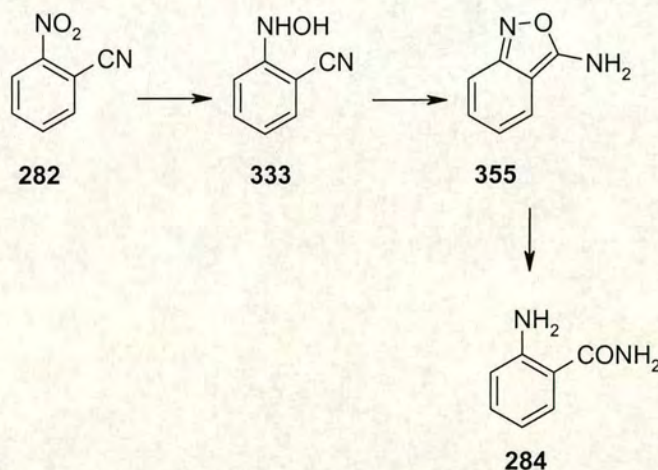


Fig 4-30 : Chemical reduction of 2-nitro-benzonitrile **282**.

The authors have discussed the possibility of tautomerisation (Fig 4-31) and have compared the N-H stretching frequencies obtained for the intermediate, with those of



heterocyclic amines which were similar in structure reported by Bellamy and Williams.<sup>151</sup> The comparison provided support that the amino tautomer **355** was the structure of the intermediate in the catalytic reduction of 2-nitro-benzonitrile **282**.

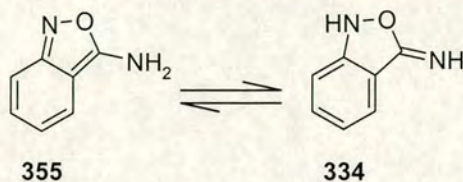


Fig 4-31 : Tautomers of 3-amino-anthranil.

The amino-anthranil **355** had a maximum UV absorption at 354 nm and was bright yellow in colour. The synthetic route to 3-amino-anthranil **355** was based on the procedure described by Musso *et al.*<sup>150</sup> 2-Nitro-benzonitrile **282** was stirred with zinc and ammonium chloride in ethanol and subsequent work-up carried out. Purification was achieved by flash-column chromatography using activated alumina to afford the 2-amino-benzamide product **284** and not 3-amino-anthranil **355** which was reported by Musso (Fig 4-32). Different purification techniques were attempted but the reported method for formation of the anthranil intermediate **355** was not reproducible, instead 2-amino-benzamide **284** was isolated each time.

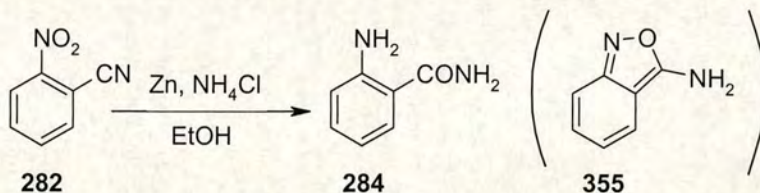


Fig 4-32 : Chemical reduction of 2-nitro-benzonitrile **282**.

The reported method was extended to 3-nitro-phthalonitrile **238** to determine whether the intermediate could be isolated. The procedure in the paper was followed but 2-amino-6-cyano-benzamide **289** was isolated and not the proposed cyclic intermediate (Fig 4-33).



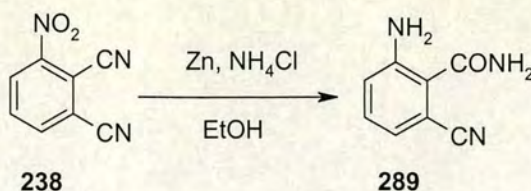


Fig 4-33 : Chemical reduction of 3-nitro-phthalonitrile **238**.

Another paper has reported on the formation of 3-amino-anthranil **355** by the electrochemical reduction of 2-nitro-benzonitrile **282**. The possibility of tautomerisation of the amino-anthranil was discussed which led to a mechanism proposed by the authors (Fig 4-34).<sup>152</sup>



Fig 4-34 : Proposed mechanism for the formation of 3-amino-anthranil **355**.

The initial step was believed to be the reduction of the nitro group in 2-nitro-benzonitrile **282** to afford the hydroxylamine intermediate **333**, which underwent intramolecular attack to form the imine compound **334**. A series of proton transfers resulted in the formation of the amino-anthranil tautomer **355**. The authors proposed that the amino form **355** was the most probable one produced in the reduction of 2-nitro-benzonitrile **282** because the imino tautomer **334** would probably be hydrolysed to **355** under the basic conditions that were employed.

It was evident from both papers,<sup>150, 152</sup> that the tautomeric state of the isolated anthranil intermediate/product **355** could not be determined by NMR or mass spectrometry. Instead structures were proposed by comparison of N-H stretch frequencies or the stability of the individual tautomer leading to the amino-anthranil **355** structure.

Boduszek *et al.* have described the synthesis of benzo-isoxazole derivatives **355** from the cleavage of amino-nitrobenzyl-phosphonates **359** in basic medium.<sup>153, 154</sup> The



treatment of 2-nitro-benzaldehyde **356** and diphenylmethylamine **357** in toluene afforded benzhydryl-(1-(2-nitro-phenyl)-methylene)-amine **358** in 98 % yield. Subsequent addition of diethyl phosphite in toluene to **358** yielded the corresponding ester as white crystals in 84 % yield (Fig 4-35). The cleavage of the diethyl phosphite ester **359** was attempted with NaOH, followed by neutralisation of the solution with HCl. However, this resulted in precipitation of the starting material **359** and not the reported 3-amino-anthranil product **355**.

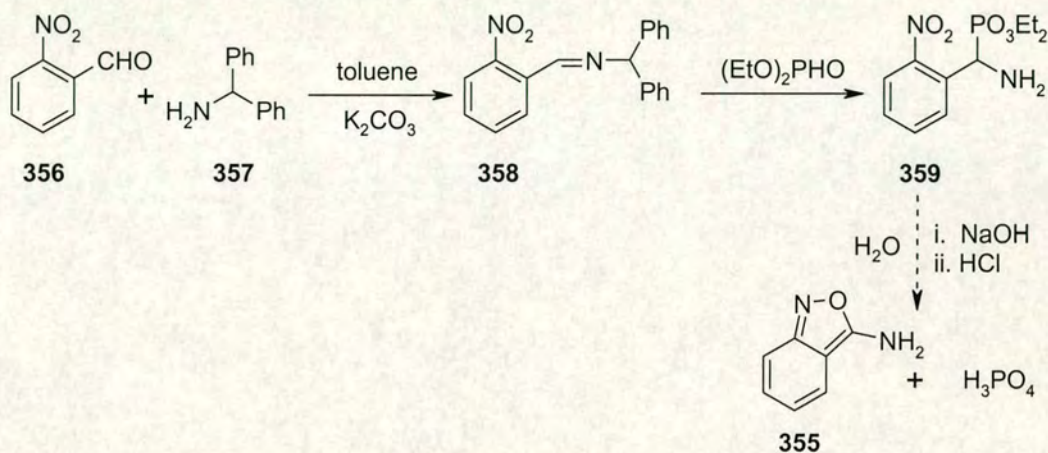


Fig 4-35 : Synthesis of 2-amino-anthranil **355**.

The structure of the tautomer **355** was further confirmed by comparison with data reported on the N-H stretching frequencies and melting points of **355** by Musso and Schröder.<sup>150</sup> Interestingly, none of the papers reported on the  $^{13}C$  NMR data of **355** which could have been compared with the NMR data obtained from the label studies of 2- $^{13}C$ -nitro-phthalonitrile **343**.

Boduszek *et al.* also assigned the structures of substituted anthranils ( $R = Bu, Bz$ ) to be the amino form **360** and not the imino tautomer **361** by comparison with the unsubstituted 3-amino-anthranil **355** based on the IR spectra and mass spectra obtained (Fig 4-36).



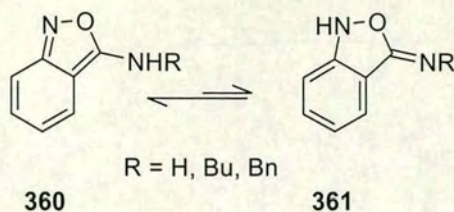


Fig 4-36 : Tautomerisation in substituted anthranils.

A recent paper by Chen *et al.* has contradicted what Boduszek *et al.* had reported on the tautomeric structures of the substituted anthranils **360**.<sup>155</sup> The structure of 3-(*N*-benzylamino)anthranil (R = Bn) was more likely to be the imino form **361** and not the amino tautomer **360** which was reported earlier. Since the maximum UV absorption of 3-amino-anthranil **355** was reported by Musso *et al.* to be 367 nm, by comparison 3-(*N*-benzylamino)-anthranil had a maximum at 312 nm. Therefore, benzylamino-anthranil lacked the 3-amino-anthranil **355** chromophore, so the structure of 3-(*N*-benzylamino)-anthranil was likely to be the imino tautomer **361**.

The maximum UV absorption of the proposed bicyclic intermediate in the reduction of 3-nitro-phthalonitrile **238** mediated by *S. cerevisiae* was investigated for comparison with the reported UV absorption of 3-amino-anthranil **355**. The reduction of 3-nitro-phthalonitrile **238** was repeated with samples removed at fixed time intervals from the reaction mixture and subjected to LCMS analysis which was attached to a diode array detector to measure UV absorption. The bicyclic intermediate had a maximum UV absorption of 386 nm and lacked the anthranil **355** chromophore of 367 nm. Therefore, the imino tautomer **337** was the likely structure of the bicyclic intermediate (Fig 4-37).



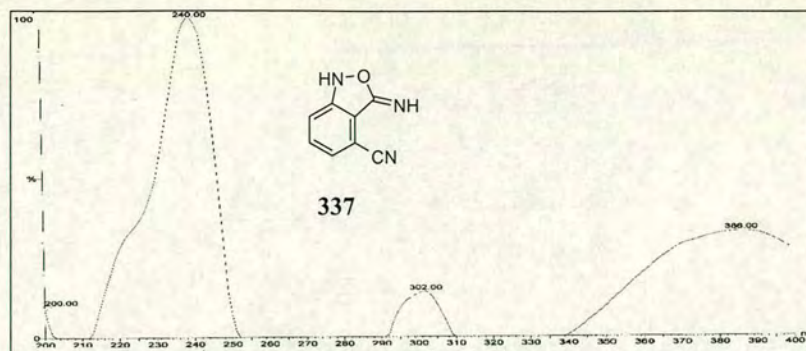


Fig 4-37: Maximum UV absorption of the proposed benzo-isoxazole intermediate **337**.

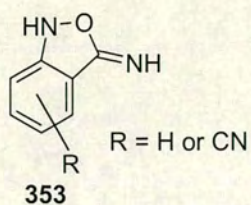
#### 4.7 Conclusion.

The intermediate detected in the reduction of dicyanonitroarenes was more likely to be the bicyclic intermediate and not the hydroxylamine compound which was evident from LCMS, GCMS, HPLC and labelling studies discussed in this chapter.

The proposed bicyclic intermediate could exist in two tautomers, the amino **354** and imino **353** form. Extensive study of the literature revealed a few papers that discussed the issue of tautomerisation in the anthranil structure. Mossu *et al.* reported on the synthesis of 3-amino-anthranil **355** which was attempted but failed. Maximum UV absorption was reported to be 367 nm and comparison of N-H stretching frequencies led to the amino tautomer **355** as the likely structure in the reduction of 2-nitro-benzonitrile **282**. The findings in this paper were compared with the work carried out by Boduszek *et al.* which was further corrected by Chen *et al.* by comparison of maximum UV absorptions.

Comparison of the maximum UV absorption reported in the literature for 3-amino-anthranil **355** was 367 nm and the bicyclic intermediate showed maximum absorption at 386 nm. Therefore, the most likely structure of the intermediate in the catalysed reduction of (di)cyanonitroarenes was the imino tautomer **353**, since it lacked the anthranil chromophore (Fig 4-38).





*Fig 4-38 :* Probable imino tautomer of benzo-isoxazole intermediate **353**.



## 5. Results & Discussion : Combined Hydrolysis & Reduction.

This chapter examines the activity of *Rhodococcus* sp. ZYL 101 towards (di)cyanonitroarene substrates. The combination of nitro-reductases (*S. cerevisiae*) and nitrilases (*Rhodococcus* sp.) has also been investigated.

### 5.1 General.

The idea was to combine the hydrolysis of the cyano group mediated by *Rhodococcus* sp. with the reduction of the nitro group carried out by *S. cerevisiae* on (di)cyanonitroarene substrates. The aim was to exploit the regio- and chemo-selectivity of two enzymes to provide access to functionalised aromatic compounds (Fig 5-1).

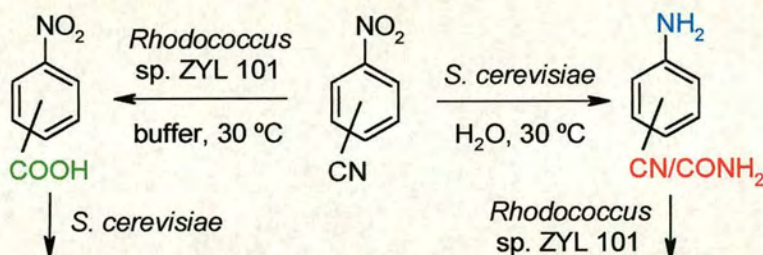


Fig 5-1 : Enzyme catalysed hydrolysis and reduction of (di)cyanonitroarenes.

The hydrolysis of nitriles was carried out using *Rhodococcus* sp. ZYL 101 (freeze-dried cells) that contained a nitrilase enzyme capable of converting nitriles to the corresponding carboxylic acids with no accumulation of the amide intermediate (Fig 5-2).

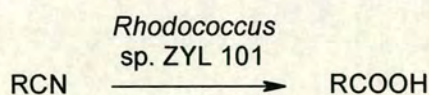


Fig 5-2 : *Rhodococcus* sp. catalysed hydrolysis of nitriles.



## 5.2 Hydrolysis of cyanonitroarenes.

Conditions of the biotransformation were optimised using 4-chloro-2-fluorobenzonitrile **362** which hydrolysed to the corresponding acid **363** in 78 % yield over 2 h when incubated with *Rhodococcus* sp. (Fig 5-3).

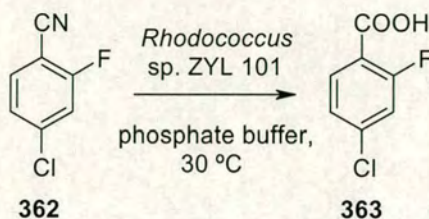


Fig 5-3 : *Rhodococcus* sp. catalysed hydrolysis of 4-chloro-2-fluorobenzonitrile **362**.

Thereafter, a range of simple cyanonitroarenes were investigated in which the cyano group was substituted either *ortho* **282**, *meta* **285** or *para* **287** to the nitro group on the aromatic ring. In each case, the cyano group was hydrolysed to afford the corresponding nitro-benzoic acids **364**, **365**, **366** in moderate yields when incubated with *Rhodococcus* sp. (Fig 5-4, Table 5-1).

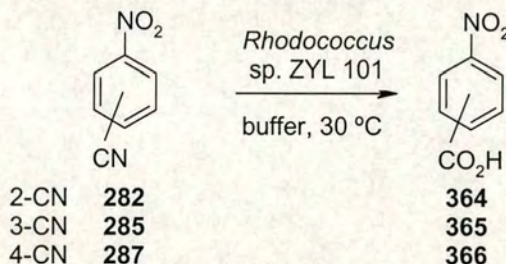


Fig 5-4 : *Rhodococcus* sp. catalysed hydrolysis of cyanonitroarenes.

Substrate		Reaction Time	Yield
<b>282</b>	<b>2-CN</b>	24 h	33 % <b>364</b>
<b>285</b>	<b>3-CN</b>	12 h	47 % <b>365</b>
<b>287</b>	<b>4-CN</b>	15 h	45 % <b>366</b>

Table 5-1 : Reaction times and yields of nitro-benzoic acids.



The progress of hydrolysis of the substrates **282**, **285**, **287** was monitored by GCMS with samples withdrawn at fixed time intervals from the reaction mixture. The substrates in which the cyano group was substituted *meta* **285** or *para* **287** to the nitro group on the aromatic ring reacted faster with moderate yields of the hydrolysed products **365**, **366** obtained in short reaction times when compared to the *ortho* cyano substrate **282**.

### 5.3 Reduction of nitro-benzoic acids.

The products **364**, **365**, **366** obtained from the hydrolysis of cyanonitroarenes **282**, **285**, **287** respectively were incubated with *S. cerevisiae*. In each case, the nitro group was reduced to the corresponding amine to afford amino acids **367**, **368**, **369** in moderate yields (Fig 5-5, Table 5-2).

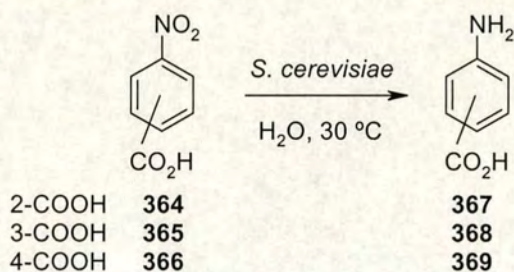


Fig 5-5 : *S. cerevisiae* catalysed reduction of nitro-benzoic acids.

Substrate		Reaction Time	Yield	
<b>364</b>	<b>2-COOH</b>	24 h	55 %	<b>367</b>
<b>365</b>	<b>3-COOH</b>	24 h	51 %	<b>368</b>
<b>366</b>	<b>4-COOH</b>	24 h	55 %	<b>369</b>

Table 5-2 : Reaction times and yields of amino benzoic acids

The amino acid products **367**, **368**, **369** were too polar to be detected by GCMS, so the progress of reduction of the substrates **364**, **365**, **366** was monitored by HPLC with samples withdrawn at fixed time intervals from the reaction mixture.



Purification was achieved by preparative HPLC (acetonitrile and water as the mobile phase) using optimised conditions for each substrate.

#### 5.4 Hydrolysis of amino-benzonitriles.

The products **283**, **286**, **288** obtained from the reduction of nitro-benzonitriles **282**, **285**, **287** respectively were incubated with *Rhodococcus* sp. over 24 h. In each case, the cyano group was hydrolysed to the corresponding carboxylic acid to afford amino acids **367**, **368**, **369**. The hydrolysis of *meta* **286** and *para* **288** cyano groups proceeded in higher yields when compared to the *ortho* cyano substrate **283** (Fig 5-6, Table 5-3).

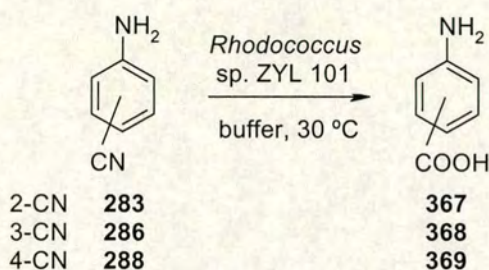


Fig 5-6 : *Rhodococcus* sp. catalysed hydrolysis of amino-benzonitriles.

Substrate		Reaction Time	Yield	
<b>283</b>	<b>2-CN</b>	24 h	35 %	<b>367</b>
<b>286</b>	<b>3-CN</b>	24 h	72 %	<b>368</b>
<b>288</b>	<b>4-CN</b>	24 h	55 %	<b>369</b>

Table 5-3 : Reaction times and yields of amino-benzoic acids.

The progress of hydrolysis of the substrates **283**, **286**, **288** was monitored by HPLC with samples withdrawn at fixed time intervals from the reaction mixture. Purification was achieved by preparative HPLC (acetonitrile and water as the mobile phase) using optimised conditions for each substrate.



2-Amino-benzamide **284** was incubated with *Rhodococcus* sp. to determine if any amide hydrolysis occurred although the *Rhodococcus* sp. contained no amidase enzyme specific for this transformation (Fig 5-7). The starting material **284** was recovered in quantitative yield after several days and it was deduced that the hydrolysis of cyanonitroarenes does not proceed through the corresponding amide, instead direct conversion of the cyano group to the carboxylic acid takes place.

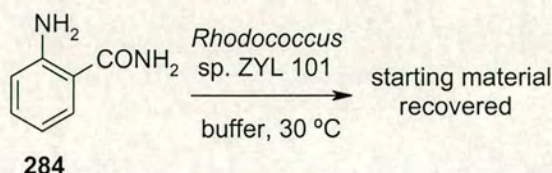


Fig 5-7 : *Rhodococcus* sp. catalysed hydrolysis of 2-amino-benzamide **284**.

## 5.5 Conclusion.

The combination of both hydrolysis and reduction on simple cyanonitroarenes was investigated to conclude whether one pathway was superior to the other. Hence, hydrolysis was carried out first followed by reduction and vice versa to deduce whether there were any significant advantages in carrying out the combined process by a specific route (Fig 5-8).

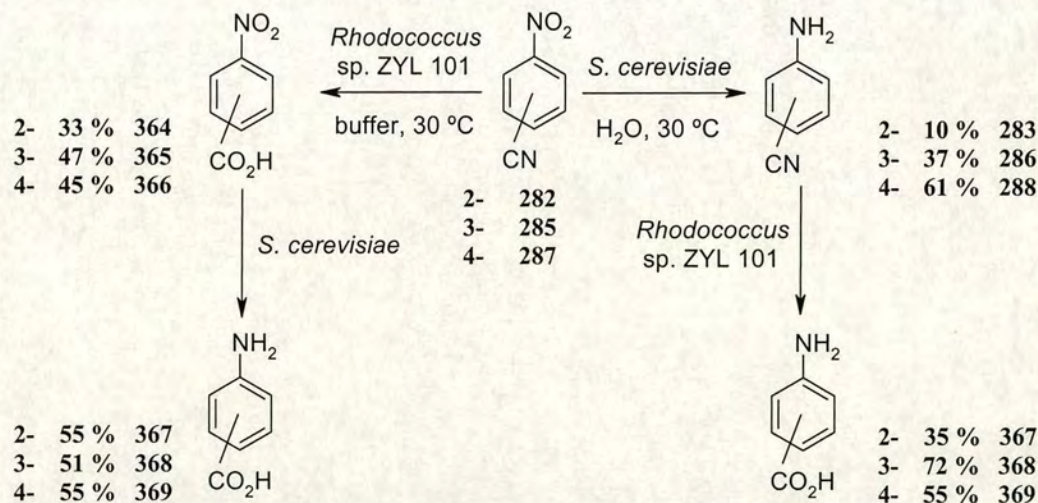


Fig 5-8 : Combined hydrolysis and reduction of cyanonitroarenes.



The same product (amino-benzoic acids) **367**, **368**, **369** was obtained in modest yields from both pathways with simple work-up procedures. It appears that there is no advantage in carrying out hydrolysis or reduction in a specific order.

## 5.6 Hydrolysis of dicyanoarenes.

Crosby *et al.* reported on the regioselective hydrolysis of substituted aromatic dicyanoarenes using *Rhodococcus* sp. that contained both a nitrilase hydratase and an amidase.<sup>156</sup> Fluorinated dicyanoarenes **370** were regioselectively hydrolysed to one regioisomer **371** in low yields (15-52 %). The cyano group that was substituted *ortho* to the amino moiety on the aromatic ring **370** was selectively hydrolysed to the amide functionality (Fig 5-9). By comparison, non-fluorinated dicyanoarenes **372** afforded mixtures of cyano acids **373**, **374** in poor regioselectivity with moderate yields (53-86 %) (Fig 5-10).

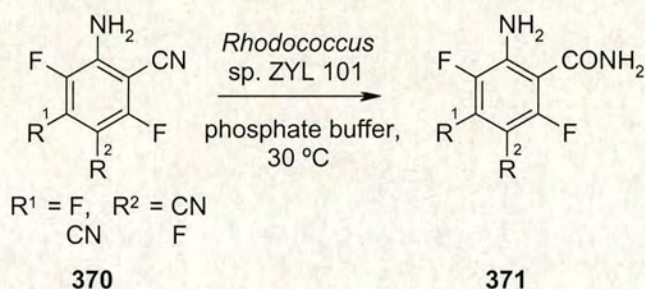


Fig 5-9 : Regioselective hydrolysis of fluorinated dicyanoarenes **370**.

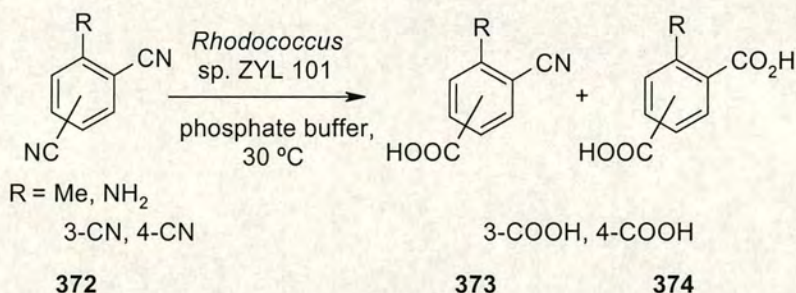


Fig 5-10 : Hydrolysis of substituted aromatic dicyanoarenes.

It was assumed that the fluorine substituents attached to the aromatic ring **370** suppressed the amidase enzyme activity present in the *Rhodococcus* sp., since the



cyano amides **371** were isolated as the final products. Whereas, both nitrilase hydratase and amidase enzymes were active in the hydrolysis of the cyano group to the corresponding mono-acids **373** and diacids **374** in non-fluorinated substrates **372**.

### 5.7 Regioselective hydrolysis of dicyanonitroarenes.

The methodology of Crosby *et al.* and the combined process of reduction and hydrolysis on simple cyanonitroarenes was extended to dicyanonitroarene substrates to exploit the regioselectivity of these processes in order to obtain functionalised substituted arenes.

The incubation of *ortho*-dicyanobenzene **335** with *Rhodococcus* sp. afforded both the diacid **375** and 2-cyano-benzoic acid **376** after 2 d in yields of 27 % and 49 % respectively (Fig 5-11).

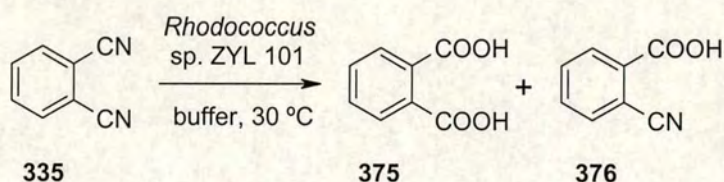


Fig 5-11 : *Rhodococcus* sp. catalysed hydrolysis of *ortho*-dicyanobenzene.

The hydrolysis of dicyanonitroarenes was investigated in those substrates **225**, **233**, **238** which had one cyano group substituted *ortho* to the nitro group and the other at either 3, 4 or 5 position to the nitro group on the aromatic ring (Fig 5-12).

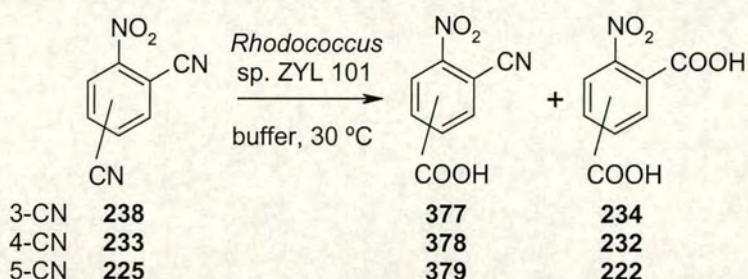


Fig 5-12 : *Rhodococcus* sp. catalysed hydrolysis of dicyanonitroarenes.



Selective hydrolysis of one cyano group substituted on the aromatic ring to the corresponding carboxylic acid occurred whereas the other remained intact to afford the mono-acids **377**, **378**, **379** with the diacid products **234**, **232**, **222** also produced. A summary of the results of the *Rhodococcus* sp. catalysed hydrolysis of dicyanonitroarenes **225**, **233**, **238** is given in Table 5-4.

Substrate		Reaction Time	Yield Mono-acid		Yield Diacid	
<b>238</b>	<b>3-CN</b>	3 d	22 %	<b>377</b>	11 %	<b>234</b>
<b>233</b>	<b>4-CN</b>	24 h	67 %	<b>378</b>	15 %	<b>232</b>
<b>225</b>	<b>5-CN</b>	10 h	70 %	<b>379</b>	15 %	<b>222</b>

Table 5-4 : Reaction times and yields of cyano-nitro-benzoic acids.

A problem arose in determining which of the two cyano functionalities had been selectively hydrolysed to obtain the mono-acids. Therefore,  $^1\text{H}$  nOe experiments were carried out on the mono-acids to determine the selectivities of the *Rhodococcus* sp. 4-Nitro-isophthalonitrile **233** was used an example to determine which regioisomer **378** or **380** was produced in the hydrolysis of this substrate **233** (Fig 5-13).

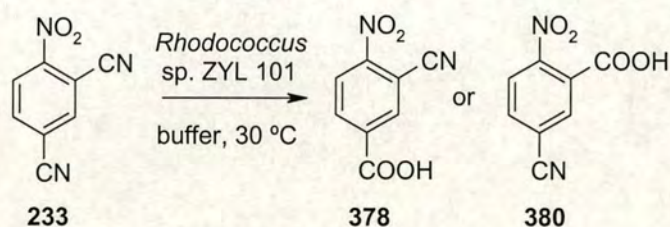


Fig 5-13 : *Rhodococcus* sp. catalysed hydrolysis of 4-nitro-isophthalonitrile **233**.

$^1\text{H}$  nOe experiments provided evidence for the structural assignment of **378**, with the irradiation of the proton of the carboxyl group signal in the  $^1\text{H}$  NMR spectrum of **378** causing an enhancement of both H<sup>1</sup> and H<sup>2</sup>. Subsequent irradiation of the carboxyl group of **380** would enhance H<sup>1</sup> only. It was deduced that the structure of the mono-



acid obtained from the *Rhodococcus* sp. catalysed hydrolysis of 4-nitro-isophthalonitrile **233** was 3-cyano-4-nitro-benzoic acid **378** (Fig 5-14).

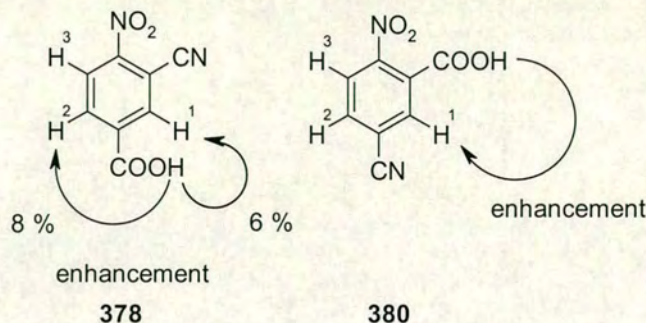


Fig 5-14 :  $^1\text{H}$  nOe enhancements caused by irradiation of the carboxyl resonance.

Further evidence for the assignment of 3-cyano-4-nitro-benzoic acid **378** was provided by comparison of the observed and theoretically calculated frequency positions of the carbon atoms of one regioisomer in the  $\delta_{\text{C}}$  NMR spectrum with the alternative regioisomeric structure (Fig 5-15).<sup>140</sup>

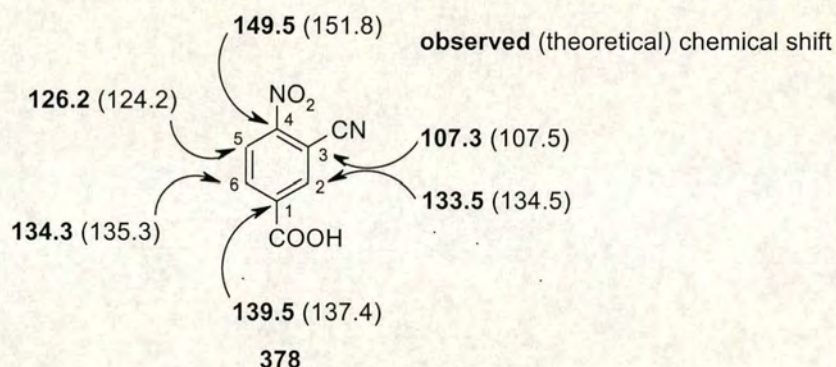


Fig 5-15 : Observed (theoretical) chemical shifts of 3-cyano-4-nitro-benzoic acid **378**.

Theoretical chemical shift calculation for the alternative structure **380** (Fig 5-16).



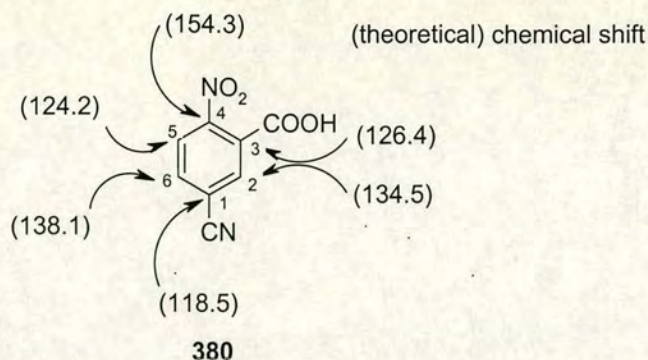


Fig 5-16 : Theoretical chemical shifts of 5-cyano-2-nitro-benzoic acid **380**.

By comparison with the calculated and observed chemical shift values, it was only possible to confidently assign C-1, C-3 and C-6 because the theoretical values of structure **378** were very similar to those observed, whereas in regioisomer **380** the theoretical chemical shifts were very different for these carbons. The theoretical chemical shifts for C-2, C-4 and C-5 were too similar in both regioisomers **378** and **380** to be useful in assigning the structure observed. Therefore, the structure of the regioisomer could be assigned on the strength of the similarity between the theoretical and observed carbon values which determined that the structure was 3-cyano-4-nitro-benzoic acid **378**.

Both <sup>1</sup>H nOe experiments and comparison of the theoretical and observed carbon chemical shifts were applied to determine the structure of the mono-carboxylic acids obtained from the hydrolysis of 3-nitro-phthalonitrile **238**, which afforded 3-nitro-2-cyano-benzoic acid **377** and 3-nitro-terephthalonitrile **225**, which afforded 3-nitro-4-cyano-benzoic acid **379** (Fig 5-17).

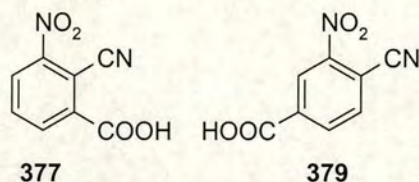


Fig 5-17 : Structures of products from the hydrolysis of 3-nitro-(tere)phthalonitrile.



Another possibility for determining the regioisomeric structures obtained from the hydrolysis of the dicyanonitroarenes substrates **225**, **233**, **238** respectively was the incubation of the hydrolysed mono-acid regioisomers **377**, **378**, **379** with *S. cerevisiae* (Fig 5-18).

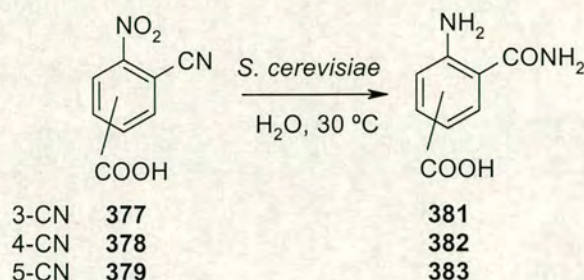


Fig 5-18 : *S. cerevisiae* catalysed reduction of cyano-nitro-benzoic acids.

*S. cerevisiae* is known to reduce the nitro group to the amino moiety in nitroarenes and also converts a cyano group substituted *ortho* to the nitro group on the aromatic ring to the amide functionality. The reduced products, **381**, **382**, **383** obtained could be characterised by IR spectroscopy which would show no CN stretch whereas the starting material (mono-acid) **377**, **378**, **379** would show the presence of a CN stretch. When the reduced products **381**, **382**, **383** were characterised, the presence of an amide functionality was prominent and no CN stretch was observed.

Alternatively, if the regioisomer **384** had no cyano group substituted *ortho* to the nitro group on the aromatic ring, then the nitro group would undergo reduction to afford the expected amino compound **385** (Fig 5-19). The reduced product(s) **385** could be characterised by IR spectrometry which would show the presence of the CN stretch.

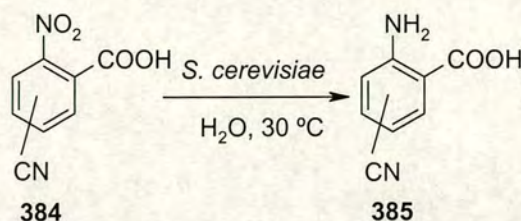


Fig 5-19 : *S. cerevisiae* catalysed reduction of cyano-nitro-benzoic acids **384**.



Thus, it was observed that positioning of the cyano group at either 3, 4 or 5 position of **225**, **233**, **238** resulted in hydrolysis to the corresponding acid **377**, **378**, **379**, whereas the cyano group that was substituted *ortho* to the nitro group remained intact.

Only one cyano group was hydrolysed in 2-nitro-isophthalonitrile **230** which had both cyano groups substituted *ortho* to the nitro group on the aromatic ring to afford **386** over a long reaction time of 6 d in 72 % yield with no diacid produced (Fig 5-20).

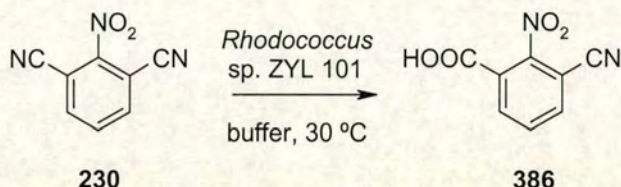
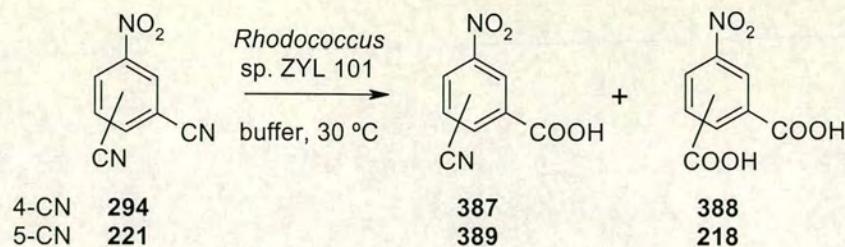


Fig 5-20 : *Rhodococcus* sp. catalysed hydrolysis of 2-nitro-isophthalonitrile **230**.

The slow rate of this reaction can be explained by the fact that cyano groups substituted *ortho* to the nitro group on the aromatic ring undergo hydrolysis very slowly. This was observed in the hydrolysis of simple cyanonitroarenes, in which *ortho*-nitro-benzonitrile **281** afforded *ortho*-nitro-benzoic acid **364** over a long reaction time (48 h), whereas those substrates which had the cyano group substituted *meta* **285** or *para* **287** to the nitro group were hydrolysed in shorter reaction times (12 h and 15 h respectively).

In the other 2 substrates, the cyano group substituted *meta* to the nitro group **221**, **294** on the aromatic ring was selectively hydrolysed to the corresponding mono-acid **387**, **389** whereas the other cyano group remained intact. However, the diacid **218**, **388** was produced as a minor impurity (Fig 5-21).



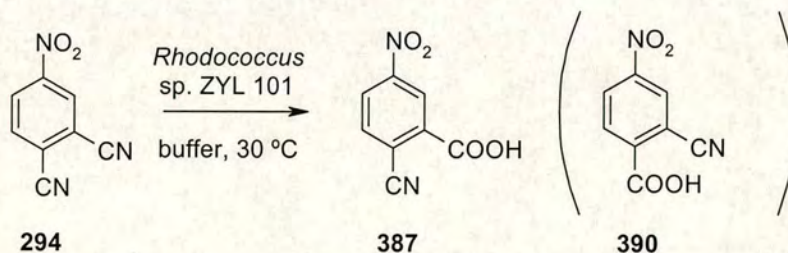
Fig 5-21 : *Rhodococcus* sp. catalysed hydrolysis of dicyanonitroarenes.

A summary of the results of the *Rhodococcus* sp. catalysed hydrolysis of substituted dicyanonitroarenes is given in Table 5-5.

Substrate		Reaction Time	Yield	
			Mono-acid	Diacid
<b>294</b>	<b>4-CN</b>	12 h	59 % <b>387</b>	8 % <b>388</b>
<b>221</b>	<b>5-CN</b>	12 h	69 % <b>389</b>	15 % <b>218</b>

Table 5-5 : Reaction times and yields of cyano-nitro-benzoic acids.

The structure of the regioisomeric compound isolated from the hydrolysis of 4-nitro-phthalonitrile **294** was determined by  $^1\text{H}$  nOe experiments and comparison of theoretical<sup>140</sup> and observed carbon chemical shift values which assigned the structure to be 3-nitro-6-cyano-benzoic acid **387** and not **390** (Fig 5-22).

Fig 5-22 : *Rhodococcus* sp. catalysed hydrolysis of 4-nitro-phthalonitrile **294**.



## 5.8 Conclusion.

Selective hydrolysis of the cyano group occurred with the following order of reactivity in dicyanonitroarene substrates:

*meta* cyano group > *para* cyano group >> *ortho* cyano group.

Selective hydrolysis of the cyano group substituted *meta* to the nitro group in 4-nitro-phthalonitrile **294** was observed whereas the *para* cyano group remained intact to afford one regioisomer **387** only. The diacid was also produced in most cases. Only one cyano group was hydrolysed in 2-nitro-isophthalonitrile **230** with a long reaction time affording the corresponding mono-acid product **386**.

## 5.9 Reduction of cyano-nitro-benzoic acids.

The mono-acid products (not the diacids) obtained from the hydrolysis of dicyanonitroarenes **221**, **225**, **230**, **233**, **238** **294** respectively were incubated with *S. cerevisiae*. The progress of reduction of all these substrates was monitored by HPLC with samples withdrawn at fixed time intervals from the reaction mixture. Purification was achieved by preparative HPLC (acetonitrile and water as the mobile phase) using optimised conditions for each substrate.

The reduction of cyano-nitro-benzoic acids was investigated in those substrates **377**, **378**, **379**, **386** which had a cyano group substituted *ortho* to the nitro group and the carboxyl functionality at either 3, 4, 5 or 6 position to the nitro group on the aromatic ring (Fig 5-23).

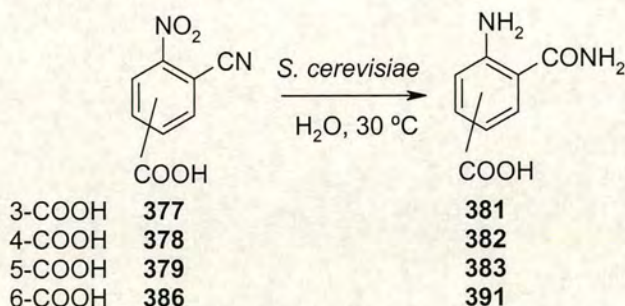


Fig 5-23 : *S. cerevisiae* catalysed reduction of cyano-nitro-benzoic acids.



The nitro group was reduced to the expected amino moiety with *S. cerevisiae* and the *ortho* cyano group was converted to the amide functionality to afford the corresponding amino acids **381**, **382**, **383**, **391**. A summary of the results of the *S. cerevisiae* catalysed reduction of substituted cyano-nitro-benzoic acids is given in Table 5-6.

Substrate	Reaction Time	Yield
<b>377</b> 3-COOH	4 d	22 % <b>381</b>
<b>378</b> 4-COOH	4 h	75 % <b>382</b>
<b>379</b> 5-COOH	3.5 h	73 % <b>383</b>
<b>386</b> 6-COOH	2 h	80 % <b>391</b>

Table 5-6 : Reaction times and yields of amino-phthalamic acids.

The conversion of the cyano group to the amide was also observed in the reduction of those (di)cyanonitroarene substrates **225**, **230**, **233**, **238** which had a cyano group substituted *ortho* to the nitro group on the aromatic ring (Fig 5-24).

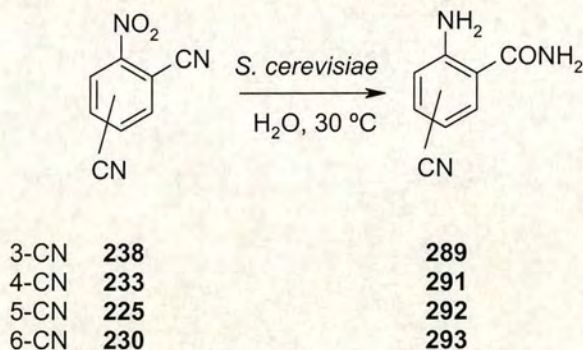


Fig 5-24 : *S. cerevisiae* catalysed reduction of dicyanonitroarene substrates.

The reduction of cyano mono-acids **377**, **378**, **379**, **386** proceeded in relatively moderate yields with short reaction times with one exception. The reduction of 2-cyano-3-nitro-benzoic acid **377** afforded 3-amino-phthalamic acid **381** in a low yield of 22 % with the reduction not gone to completion (Fig 5-25).



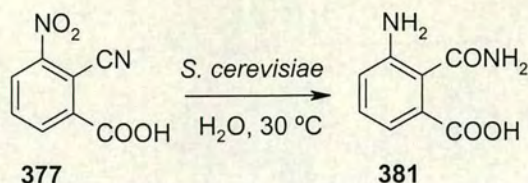


Fig 5-25 : *S. cerevisiae* catalysed reduction of 2-cyano-3-nitro-benzoic acid **377**.

No amide functionality was observed in those products **392**, **393** obtained from the reduction of cyano-nitro-benzoic acid substrates **387**, **389** which had no cyano group substituted *ortho* to the nitro group on the aromatic ring (Fig 5-26).

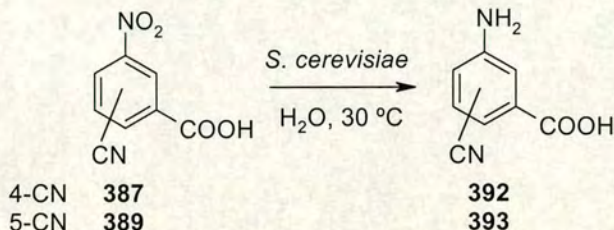


Fig 5-26 : *S. cerevisiae* catalysed reduction of cyano-nitro-benzoic acids.

The expected reduced products **392**, **393** were obtained by preparative HPLC in moderate yields but longer reaction times were required for complete substrate consumption compared to the *ortho*-cyano substrates **377**, **378**, **379**, **386** (Table 5-7).

Substrate	Reaction Time	Yield
<b>387</b> 4-CN	8 h	61 % <b>392</b>
<b>389</b> 5-CN	6 h	60 % <b>393</b>

Table 5-7 : Reaction times and yields of amino-cyano-benzoic acids.

## 5.10 Conclusion.

Complete substrate consumption occurred in short reaction times to afford amino-phthalamic acids **381**, **382**, **383**, **391** from cyano-nitro-benzoic acids **377**, **378**, **379**, **386** that had a cyano group substituted *ortho* to the nitro group. Whereas, the expected amino-cyano-benzoic acids **392**, **393** were produced in longer reaction



times in the dicyano substrates **387**, **389** which had no cyano group substituted *ortho* to the nitro group on the aromatic ring.

### 5.11 Hydrolysis of amino-cyano-benzamides.

The products obtained from the *S. cerevisiae* catalysed reduction of dicyanonitroarenes **221**, **225**, **230**, **233**, **238** **294** respectively were incubated with *Rhodococcus* sp. to determine whether any hydrolysis occurred. In each case, the cyano group was hydrolysed to the carboxyl group in the isomeric substrates **289**, **291**, **292**, **293** which had an amide moiety substituted *ortho* to nitro group and the cyano group present at either 3, 4, 5 or 6 position to the nitro group on the aromatic ring to afford amino-phthalamic acids (Fig 5-27).

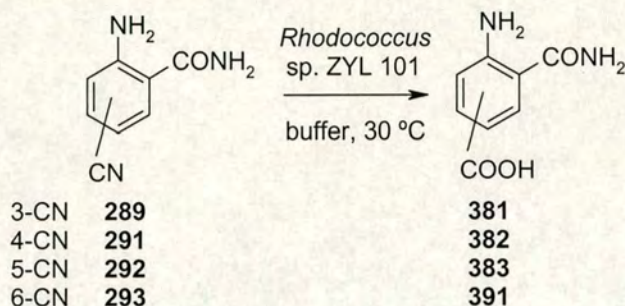


Fig 5-27 : *Rhodococcus* sp. catalysed hydrolysis of amino-cyano-benzamides.

The progress of hydrolysis of the substrates **289**, **291**, **292**, **293** was monitored by HPLC with samples withdrawn at fixed time intervals from the reaction mixture. Purification was achieved by preparative HPLC (acetonitrile and water as the mobile phase) using optimised conditions for each substrate. A summary of the results of the *Rhodococcus* sp. catalysed reduction of substituted amino-cyano-benzamides is given in Table 5-8.



Substrate		Reaction Time	Yield
<b>289</b>	<b>3-CN</b>	48 h	12 % <b>381</b>
<b>291</b>	<b>4-CN</b>	24 h	67 % <b>382</b>
<b>292</b>	<b>5-CN</b>	24 h	70 % <b>383</b>
<b>293</b>	<b>6-CN</b>	3 d	89 % <b>391</b>

Table 5-8 : Reaction times and yields of amino-phthalamic acids.

The amide group of 2-amino-6-cyano-benzamide **289** was hydrolysed to the corresponding acid **290** when incubated in the buffer/enzyme suspension which resulted in a very low yield of the expected hydrolysed 3-amino-phthalamic acid product **381** (12 %) (Fig 5-28).

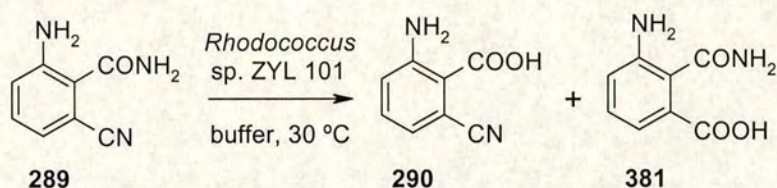


Fig 5-28 : *Rhodococcus* sp. catalysed hydrolysis of 2-amino-6-cyano-benzamide **289**.

The hydrolysis of the amide group was also observed in the reduction of 3-nitro-phthalonitrile **238**, in which the reduced product 2-amino-6-cyano-benzamide **289** was hydrolysed to 2-amino-6-cyano-benzoic acid **290** under *S. cerevisiae* conditions (Fig 5-29).

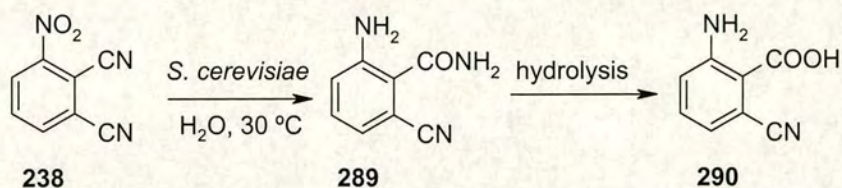


Fig 5-29 : *S. cerevisiae* catalysed reduction of 3-nitro-phthalonitrile **238**.



The cyano group substituted *meta* to the amino group **295**, **296** on the aromatic ring was selectively hydrolysed to the corresponding mono-acid **392**, **393** whereas the other cyano group remained intact. However, the diacid **394**, **395** was produced as a minor impurity (Fig 5-30).

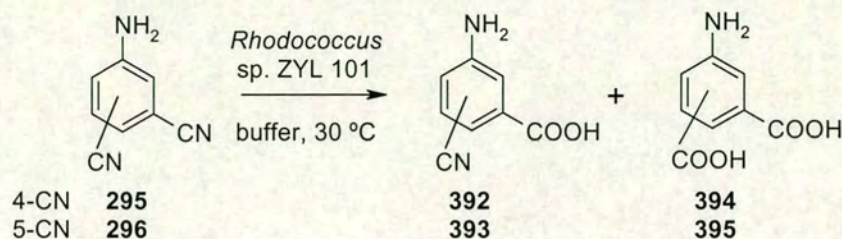


Fig 5-30 : *Rhodococcus* sp. catalysed hydrolysis of dicyanoaminoarenes.

The products (mono-acids) **392**, **393** were obtained in moderate yields along with the diacids **394**, **395** in longer reaction times compared to those amino-cyano-benzamide substrates **289**, **291**, **292**, **293** which had an amide group substituted *ortho* to the nitro group on the aromatic ring. A summary of the results of the *Rhodococcus* sp. catalysed hydrolysis of substituted dicyanoaminoarenes **295**, **296** is given in Table 5-9.

Substrate		Reaction Time	Yield Mono-acid	Yield Diacid
<b>295</b>	<b>4-CN</b>	48 h	53 % <b>392</b>	14 % <b>394</b>
<b>296</b>	<b>5-CN</b>	3 d	49 % <b>393</b>	16 % <b>395</b>

Table 5-9 : Reaction times and yields of amino-cyano-benzoic acids.

The structure of the product **392** isolated from the hydrolysis of 4-amino-phthalonitrile **295** was determined by  $^1\text{H}$  nOe experiments and comparison of theoretical<sup>140</sup> and observed carbon chemical shift values which assigned the structure to be 3-amino-6-cyano-benzoic acid **392** and not **396** (Fig 5-31).



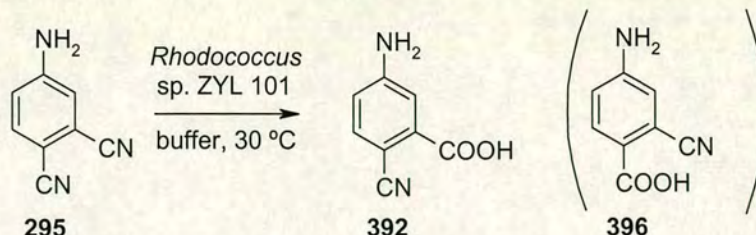


Fig 5-31 : *Rhodococcus* sp. catalysed hydrolysis of 4-amino-phthalonitrile **295**.

## 5.12 Conclusion.

The amino-phthalamic acids **381**, **382**, **383**, **391** were obtained in moderate yields with short reaction times in those amino-cyano-benzamide substrates **289**, **291**, **292**, **293** which had an amide group substituted *ortho* to the nitro group on the aromatic ring. Conversely, the mono-acid products (amino-cyano-benzoic acids) **392**, **393** as well as the diacids were obtained in longer reaction times from the hydrolysis of dicyanoaminoarenes **295**, **296**.

## 5.13 Enzyme catalysed reduction and hydrolysis of aromatic arenes.

The aim was to exploit the selectivity of both whole-cell systems (*S. cerevisiae* and *Rhodococcus* sp.) to obtain products of interest which would be very difficult to achieve using traditional chemical methodology.

### 5.13.1 Reduction and hydrolysis of 3-nitro-phthalonitrile **238**.

The reduction of 3-nitro-phthalonitrile **238** afforded the corresponding 2-amino-6-cyano-benzamide product **289** (50 %) which was further hydrolysed to afford 3-amino-phthalamic acid **381** in a low yield of 12 % in 48 h and **290** (impurity) (Fig 5-32). If the hydrolysis step was carried out first, selective hydrolysis of the *meta* cyano group occurred to afford 3-nitro-6-cyano-benzoic acid **377** (22 %) along with the diacid product **234**. Purification by preparative HPLC was carried out to isolate



the mono-acid which was then reduced to afford 3-amino-phthalamic acid **381** in a yield of 22.0 % in 4 d which was the same product as that obtained if the order of reactions was reversed.

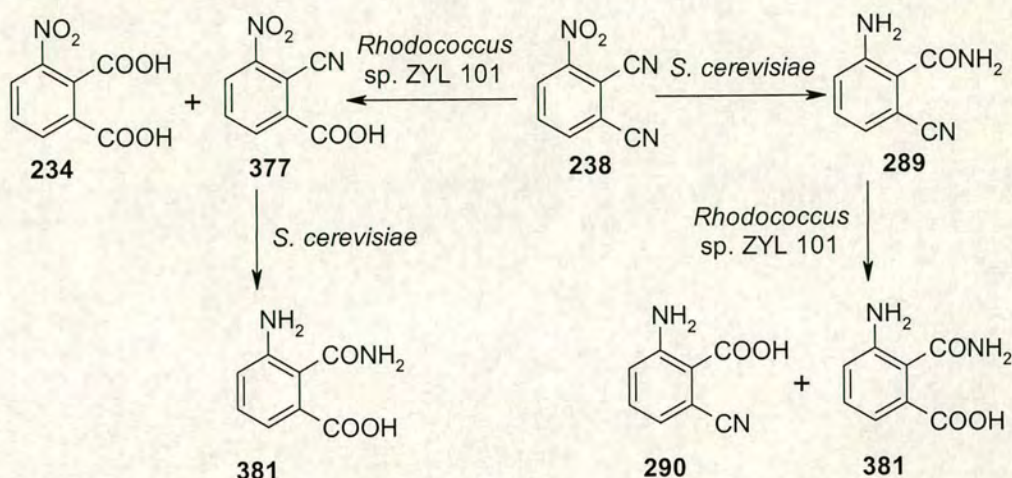


Fig 5-32 : Combined hydrolysis and reduction of 3-nitro-phthalonitrile **238**.

Therefore, not a significant difference in both pathways, since the same product was obtained in low yields. Purification by preparative HPLC was required for both routes, if the hydrolysis was carried out first, the mono-acid and diacid had to be purified to carry out the next step. If reduction was the first step followed by the hydrolysis step, the phthalamic acid and the minor impurity had to be purified otherwise both routes are feasible with low yields obtained at the end.

### 5.13.2 Reduction and hydrolysis of 3-nitro-tere- and 4-nitro-iso-phthalonitrile **233**, **225**.

This class of substrates have one cyano group substituted at either 4 or 5 position to the nitro group and the other one substituted *ortho* to the nitro group on the aromatic ring. With both substrates, 4-nitro-isophthalonitrile **233** and 3-nitro-terephthalonitrile **225**, very similar moderate yields and reaction times were obtained for each product and therefore for each reaction step (Fig 5-33).



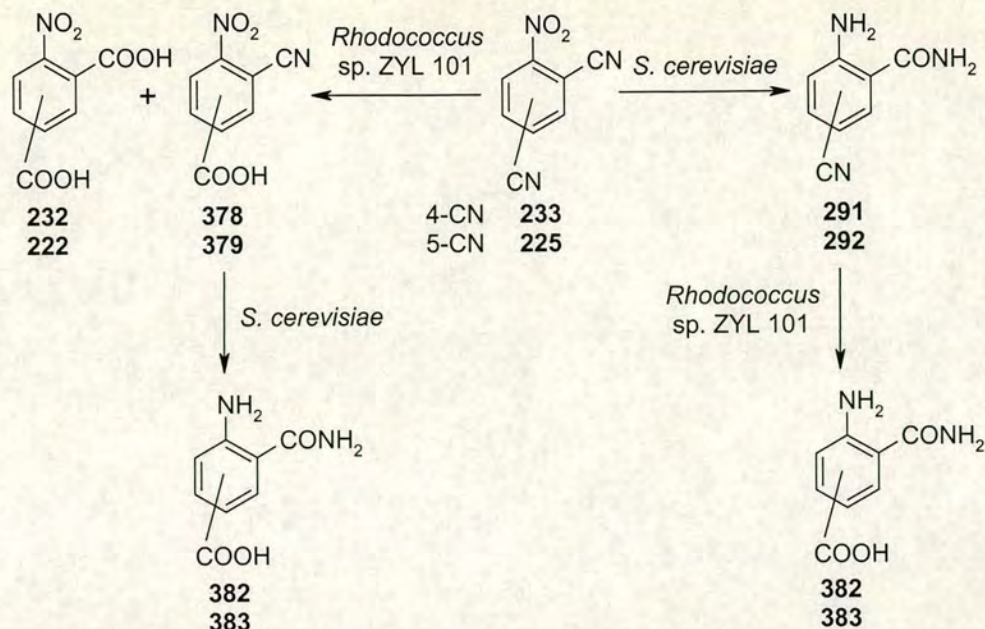


Fig 5-33 : Combined hydrolysis and reduction of dicyanonitroarenes **233**, **225**.

Generally, if the reduction was carried out first, the nitro and the *ortho* cyano group underwent reduction and conversion to afford amino-cyano-benzamide derivatives **292**, **292** and then subsequent hydrolysis afforded the corresponding amino-phthalamic acids **382**, **383** (67 %, 70 % yields respectively) which were purified by preparative HPLC. Whereas, the hydrolysis of the dicyanonitroarene substrates **233**, **225** afforded a mixture of products, the mono-acid **378**, **379** and the diacid **232**, **222** which were purified by preparative HPLC. Then further reduction yielded the amino-phthalamic acids **382** and **383** (75 %, 73 % yields respectively) which were obtained by preparative HPLC.

In this reaction scheme, although both reaction pathways afforded moderate yields of the same product(s), reduction followed by hydrolysis would be more favoured since it involves a simple work-up procedure. Reduction of the substrate yields one product which was purified by flash-column chromatography followed by hydrolysis to yield another product which was purified by preparative HPLC. In contrast, the hydrolysis of the substrate afforded a mixture of products which needed to be isolated by preparative HPLC in order to carry out the next reduction step. In this



reduction step, the mono-acid was converted to the amino-phthalamic acid which was also purified by preparative HPLC.

### 5.13.3 Reduction and hydrolysis of 2-nitro-isophthalonitrile **230**.

The reduction of 2-nitro-isophthalonitrile **230** yielded the expected 2-amino-3-cyano-benzamide product **293** (74 %) which was further hydrolysed to afford 2-amino-isophthalamic acid **391** in a low yield of 22 % and a long reaction time of 4 d (Fig 5-34). In contrast carrying out the hydrolysis first resulted in the hydrolysis of one cyano group to afford 3-cyano-2-nitro-benzoic acid **386** in a high yield of 72 % after 5 h and then subsequent reduction afforded the same product **391** in 89 % yield.

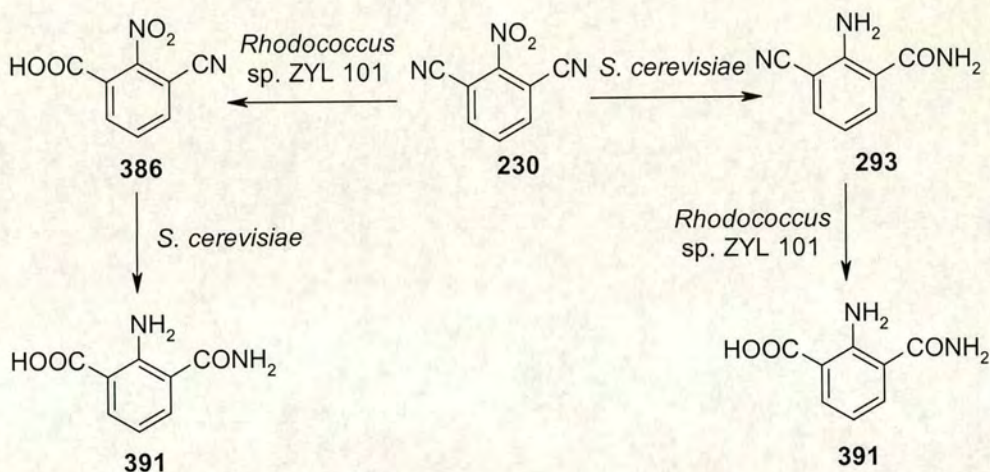


Fig 5-34 : Combined hydrolysis and reduction of 2-nitro-isophthalonitrile **230**.

Hydrolysis of the dicyanonitroarene followed by reduction would be a more favourable route because the reaction time is short with the yield of the end product obtained much higher (89 %) compared to the yield of the same product (22 %) obtained if the order of reactions was reversed.



### 5.13.4 Reduction and hydrolysis of 5-nitro- and 4-nitro-iso-phthalonitrile 221, 294.

This class of substrates have one cyano group substituted at either 4 or 5 position to the nitro group and the other one substituted *meta* to the nitro group on the aromatic ring. With both substrates, 4-nitro-phthalonitrile **294** and 5-nitro-isophthalonitrile **221**, very similar moderate yields and reaction times were obtained for each product and therefore for each reaction step (Fig 5-35).

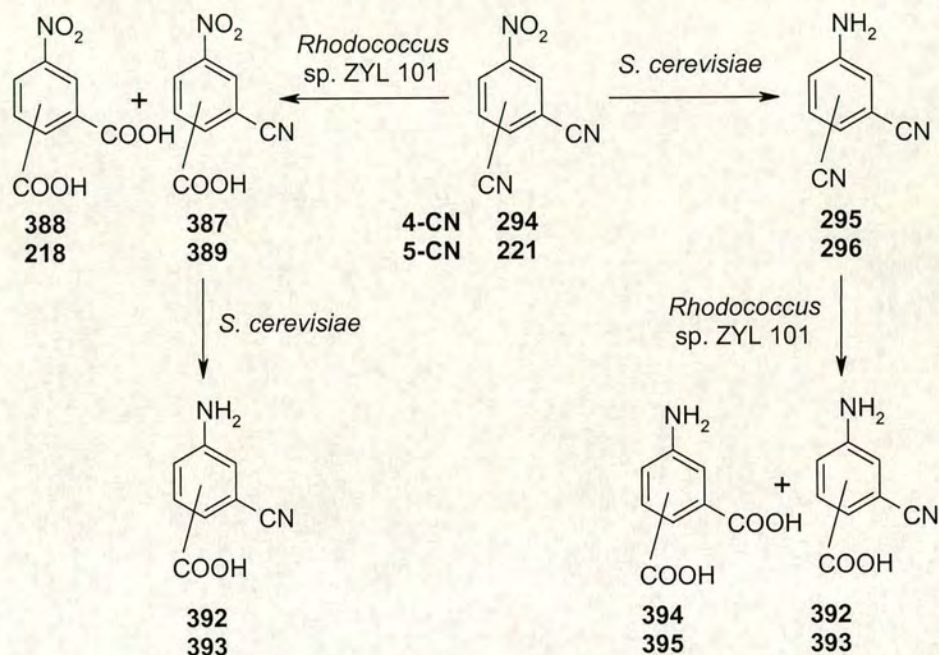


Fig 5-35 : Combined hydrolysis and reduction of dicyanonitroarenes.

Reduction of the dicyanonitroarenes **294** and **221** afforded the expected amino-(iso)phthalonitrile products **295** and **296** followed by hydrolysis to yield a mixture of products which were isolated by preparative HPLC affording the corresponding amino-cyano-benzoic acids **392**, **393** in moderate yields (53 %, 49 % respectively). If hydrolysis was the first step, two hydrolysed products were obtained, with the mono-acids **387**, **389** isolated from the diacids **388**, **218** by preparative HPLC. Subsequent reduction afforded the same amino-cyano-benzoic acids **392**, **393** by preparative HPLC in moderate yields (60 %, 61 % respectively).



There is no significant difference in both pathways, since the same product was obtained in moderate yields and similar reaction times. An extra work-up step involving purification by preparative HPLC was required if the hydrolysis was carried out first, since the mono-acid and diacid had to be purified, otherwise both routes are feasible with the same product(s) obtained.

#### **5.14 Conclusion.**

Starting with a dicyano-nitro aromatic compound and producing a tri-substituted aromatic ring where all three functional groups are different has been achieved under biological conditions. Under chemical conditions, it would be difficult to distinguish one cyano group from another. We have shown that by combining the processes of two whole-cell systems it was possible to selectively distinguish one cyano group from another.

In some substrates, the product was an aromatic amino acid which may have applications in pharmaceutical research. Regioselectivity of certain reaction steps during drug synthesis is an important issue and we have shown one method of overcoming this problem by using whole-cell systems.

It would be interesting to know which whole-cell system is superior or will dominate its effect when both *S. cerevisiae* and *Rhodococcus* sp. is added to simple nitro aromatic compounds simultaneously.



## 6. Experimental : Synthesis.

### 6.1 General experimental.

#### (a) Instrumentation.

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on Bruker AC250, WH360 or Varian Gemini 200 instruments. Both  $^1\text{H}$  and  $^{13}\text{C}$  spectra were referenced using residual protic solvents either  $(\text{CD}_3)_2\text{SO}$  [ $\delta_{\text{H}}$  2.5, q;  $\delta_{\text{C}}$  39.7, m] or  $\text{CDCl}_3$  [ $\delta_{\text{H}}$  7.26, s;  $\delta_{\text{C}}$  77.0, t] or  $(\text{CD}_3)_2\text{CO}$  [ $\delta_{\text{H}}$  2.05, q;  $\delta_{\text{C}}$  29.8, m]. Chemical shifts ( $\delta_{\text{H}}$ ,  $\delta_{\text{C}}$ ) are recorded in parts per million (ppm) and coupling constants ( $J$ ) are measured in Hertz (Hz) and quoted to the nearest 0.5 Hz.

Infrared spectroscopy was recorded on a Perkin Elmer Paragon 1000 FT-IR spectrophotometer with the frequencies ( $\nu$ ) measured in wavenumbers ( $\text{cm}^{-1}$ ). Infrared spectra were recorded either as thin films on sodium chloride plates or as KBr disks and are denoted by (film) or (KBr) respectively.

Melting points were obtained on a Gallenkamp melting point apparatus and are quoted in degrees Celsius ( $^{\circ}\text{C}$ ) and uncorrected.

Elemental analysis (CHN) was performed using a Perkin Elmer 2400 CHN Elemental Analyser.

UV/Visible Spectroscopy was performed on either a Hewlett Packard 8453 or Unicam UV2-100 UV/Visible Spectrometer.

Fast atom bombardment (FAB) was performed using a Kratos MS50TC instrument, Electrospray (ES) and Atmospheric Pressure Chemical Ionisation (CI) mass spectra were recorded on a Micromass Platform II and Electron Impact (EI) ionisation was carried out on a Finnegan 4500 instrument. All are quoted as Daltons.



**(b) Chromatography.**

Analytical thin layer chromatography (TLC) was carried out on Merck aluminium backed plates coated with silica gel 60 F<sub>254</sub> (0.25 mmol). The components were visualised using ultra-violet light (245 nm), potassium permanganate and ammonium molybdate dips.

Flash-column chromatography was carried out using the appropriate sized parallel-sided column filled with silica gel 60 (Merck, particle size 0.04-0.063 nm) unless stated otherwise. Eluent compositions are quoted as v/v ratios.

Neutral alumina (50-200 micron, 100-200 mesh, activity III) was purchased from Acros and deactivated to Brockmann grade III with addition of water.<sup>157</sup>

**(c) Solvents and Reagents.**

All solvents and reagents were standard laboratory grade and used as supplied unless otherwise stated. Tetrahydrofuran was pre-dried over sodium wire and distilled from sodium benzophenone ketal while dichloromethane was distilled from calcium hydride and other solvents were purchased as anhydrous grade. Petroleum ether is referred to as petrol, Bp 45-60 °C.

Resin (AG 50W x 2, 100-200 mesh H<sup>+</sup>) was purchased from Bio-Rad and was washed several times with dilute HCl (2M), filtered and washed with deionised water prior to use.

**(d) Gas Chromatography Mass Spectroscopy (GCMS).**

GCMS chromatographs were recorded on a Hewlett Packard (HP) 6890 series Gas Chromatograph instrument and HP 597 Mass Selective Detector using a HP-5 capillary column (crosslinked 5 % phenyl methyl siloxane, 30 m by 0.32 mmol by



0.25  $\mu$ L film thickness). The HP ChemStation (Version B.02.05) software package was used to analyse the results.

Conditions: Inlet Heater 250 °C, Pressure psi 13.3, Total flow 1.00 mL/min  
Oven variable.

**(e) High Performance Liquid Chromatography (HPLC).**

HPLC analysis was carried out using a Waters 486 Tunable Absorbance Detector and a Waters 600 Pump and Controller. The results were analysed with the Waters Millennium Chromatography Manager software package.

Chiral HPLC analysis was achieved using a Chiracel OD column as the stationary phase eluting with either hexane:*iso*-propanol or hexane:ethanol with a flow rate of 0.60 mL/min unless otherwise.

Analytical HPLC was achieved using a Phenomenex Spherclone ODS 2 column (5 $\mu$ m particle size; dimensions: 25 cm by 4.60 mm) eluting with acetonitrile:water (0.1 % TFA) with a flow rate of 1 mL/min.

Preparative HPLC was achieved using a Phenomenex Spherclone ODS 2 column (5 $\mu$  particle size; dimensions: 250 by 10.00 mm) eluting with acetonitrile:water 0.1 % TFA) with a flow rate of 4.7 mL/min.

**(f) Liquid Chromatography Mass Spectroscopy (LCMS).**

LCMS studies were carried out on a Micromass Platform using a Waters 486 UV detector and a Waters Alliance 2690 Pump and controller. Masslynx (Version 2.3) software package was used to analyse the results.

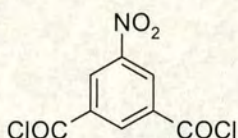


Photodiode array studies were carried out on a Micromass ZMD 4000 spectrometer using a Waters 966 Photodiode Array Detector and Waters 600 Pump with Masslynx software (Version 3.3).

Sample preparation: 1 mL of the reaction mixture was removed and microcentrifuged. A sample of the aqueous layer (200  $\mu$ L) was removed, filtered and diluted with water:acetonitrile (800  $\mu$ L; 0.1 % TFA; 1:1).

## 6.2 Synthesis of dicyanonitroarenes.

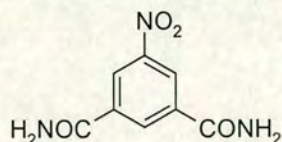
### 5-Nitro-isophthaloyl dichloride **219**.<sup>158</sup>



Under an inert atmosphere, 5-nitro-isophthalic acid **218** (15.00 g, 71.0 mmol, pre-dried at 100 °C for 1 h) was heated under reflux with thionyl chloride (200.00 g, 122.7 mL) for *ca.* 48 h or until all the acid had dissolved. Excess thionyl chloride was removed *in vacuo* to yield an oil which afforded on cooling white needle-like crystals of 5-nitro-isophthaloyl dichloride **219** (17.10 g, 97.1 %).

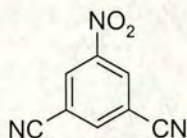
Mp 66.0-67.5 °C, Lit.<sup>159</sup> 67.5-68.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3088 (CH), 1756 (C=O), 1615 (Ph), 1535, 1349 (NO<sub>2</sub>);  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 200 MHz) 9.21 (2H, d, *J* 1.8, PhH), 9.10 (1H, t, *J* 1.8, PhH);  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 63 MHz) 165.5, 137.2 (C), 135.9 (CH), 134.2 (C), 130.7 (CH); *m/z* (EI) 248 (M<sup>+</sup>), 221 (M-HCN), 202 (M-NO<sub>2</sub>).



**5-Nitro-isophthalamide 220.**<sup>160</sup>

5-Nitro-isophthaloyl dichloride **219** (15.00 g, 60.0 mmol) was introduced slowly into a cooled (0 °C) beaker of concentrated ammonia solution (30 mL) and the resulting heterogeneous mixture stirred at 25 °C for 30 min. The diamide was collected by gravity filtration, dried *in vacuo* and the resulting solid purified by recrystallisation (water) to afford 5-nitro-isophthalamide **220** as white crystals (10.80 g, 85.6 %).

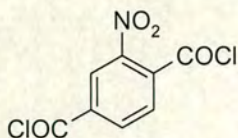
Mp 310.0-311.3 °C, Lit.<sup>161</sup> 312.0-314.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3345, 3207 (NH<sub>2</sub>), 1715 (C=O), 1682 (NH), 1613 (Ph), 1520, 1353 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.91 (1H, t, *J* 1.5, PhH), 8.80 (2H, d, *J* 1.5, PhH), 8.52 (4H, br s, CONH<sub>2</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 165.5, 148.1, 136.2 (C), 132.8, 124.8 (CH); *m/z* (CI<sup>+</sup>) 210 (MH<sup>+</sup>), 179 (M-NO).

**5-Nitro-isophthalonitrile 221.**<sup>162</sup>

5-Nitro-isophthalamide **220** (7.06 g, 33.8 mmol, pre-dried at 110 °C for 2 h) was heated under reflux with excess phosphorus oxychloride (*ca.* 23 mL) for 4 h. The resulting yellow solution was concentrated under reduced pressure to yield a light brown solid. Recrystallisation (benzonitrile) afforded 5-nitro-isophthalonitrile **221** as light brown crystals (2.80 g, 49.9 %).

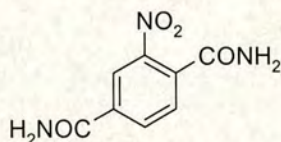
*R<sub>f</sub>* (EtOAc:Petrol, 1:1) 0.56; Mp 205.0-208.5 °C, Lit.<sup>162</sup> 205.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3079 (CH), 2240 (CN), 1608 (Ph), 1544, 1355 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.06 (2H, d, *J* 1.5, PhH), 8.09 (1H, t, *J* 1.5, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 148.2 (C), 142.2, 131.9 (CH), 115.8, 144.2 (C); *m/z* (EI) 173 (M<sup>+</sup>), 143 (M-NO), 127 (M-NO<sub>2</sub>), 100 (M-NO<sub>2</sub>-HCN); GC: *R<sub>t</sub>* 6.5 min, oven temp 150 °C.



**2-Nitro-terephthaloyl dichloride 223.**<sup>163</sup>

Nitro-terephthalic acid **222** (21.10 g, 0.1 mmol, pre-dried using phosphorus pentoxide for 1 h) was stirred vigorously with phosphorus pentachloride (50.00 g, 0.2 mmol). The liquid was heated at 90 °C for 1 h, cooled and the product extracted with hexane (3 x 25 mL). The combined organic extracts were concentrated *in vacuo* to afford the *acid dichloride 223* as light yellow oil (20.37 g, 82.1 %).

Bp 139.0 °C, Lit.<sup>164</sup> 140.0-142.0 °C;  $\nu_{\max}(\text{film})/\text{cm}^{-1}$  3096 (CH), 1773 (C=O), 1619 (Ph), 1543, 1349 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{CO}, 200 \text{ MHz})$  8.85 (1H, d, *J* 1.8, PhH), 8.72 (1H, dd, *J* 8.1 and 1.8, PhH), 8.13 (1H, d, *J* 8.1, PhH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{CO}, 63 \text{ MHz})$  204.8, 200.6 (C), 134.4 (CH), 134.2, 132.8, 129.6 (C), 128.2, 124.7 (CH); *m/z* (CI<sup>+</sup>) 249 (MH<sup>+</sup>), 218 (M-NO), 202 (M-NO<sub>2</sub>).

**2-Nitro-terephthalamide 224.**<sup>165</sup>

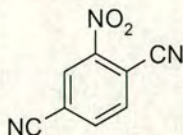
2-Nitro-terephthaloyl dichloride **223** (20.00 g, 80.6 mmol) was introduced slowly into a cooled (0 °C) beaker of concentrated ammonia solution (40 mL) and the resulting heterogeneous mixture stirred at 25 °C for 24 h. The diamide was collected by gravity filtration, dried *in vacuo* and the resulting solid purified by recrystallisation (water) to afford *2-nitro-terephthalamide 224* as yellow needle-like crystals (15.88 g, 94.2 %).

Mp 273.0-275.4 °C, Lit.<sup>165</sup> 275.0-276.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3359, 3197 (NH<sub>2</sub>), 1663 (C=O, NH), 1680 (Ph), 1533, 1350 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{SO}, 200 \text{ MHz})$  8.45 (1H, d, *J* 1.7, PhH), 8.35 (1H, br s, CONH), 8.25 (1H, br s, CONH), 8.22 (1H, dd, *J* 7.9 and 1.7, PhH), 7.81 (1H, br s, CONH), 7.78 (1H, br s, CONH), 7.72 (1H, d, *J* 7.9, PhH);



$\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 166.9, 165.4, 147.1, 136.2, 135.0 (C), 132.3, 129.3, 123.1 (CH);  $m/z$  (ES<sup>+</sup>) 210 (MH<sup>+</sup>), 192 (M-NH<sub>3</sub>), 179 (M-NO).

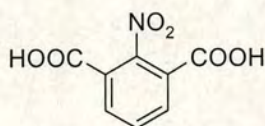
### 2-Nitro-terephthalonitrile **225**.<sup>166</sup>



2-Nitro-terephthalamide **224** (7.65 g, 36.6 mmol, pre-dried at 110 °C for 2 h) was heated under reflux with excess phosphorus oxychloride (*ca.* 25 mL) for 4 h. The resulting yellow solution was concentrated under reduced pressure to yield a light brown solid. Recrystallisation (water) afforded 2-nitro-terephthalonitrile **225** as beige crystals (2.40 g, 37.1 %).

$R_f$  (EtOAc) 0.24; Mp 125.7-127.2 °C, Lit.<sup>166</sup> 124.0-126.0 °C;  $\nu_{\text{max}}$ (KBr)/cm<sup>-1</sup> 3088 (CH), 2242 (CN), 1625 (Ph), 1543, 1356 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.95 (1H, d,  $J$  1.5, PhH), 8.47 (1H, dd,  $J$  8.1 and 1.5, PhH), 8.39 (1H, d,  $J$  8.1, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 148.7 (C), 138.3, 136.7, 129.6 (CH), 116.7, 116.2, 114.8, 111.2 (C);  $m/z$  (EI) 173 (M<sup>+</sup>), 143 (M-NO), 127 (M-NO<sub>2</sub>), 100 (M-NO<sub>2</sub>-HCN); GC:  $R_t$  9.7 min, oven temp 150 °C.

### 2-Nitro-isophthalic acid **227**.<sup>167</sup>



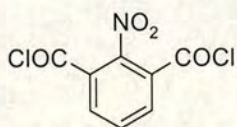
1,3-Dimethyl-2-nitrobenzene **226** (20.00 g, 0.13 mmol, 18 mL), distilled water (400 mL) and potassium permanganate (40.20 g, 2.5 eq.) were placed in a 3-necked flask fitted with a thermometer, a magnetic stirrer and a reflux condenser. The resulting mixture was stirred vigorously for 5 min, then heated to 85 °C after which the internal temperature was maintained at 80 °C using an ice bath to control the initial exothermic reaction. Upon completion the solution was heated under reflux for 2 h at 90 °C, cooled to 75 °C and filtered through a bed of Celite<sup>TM</sup>. The yellow



filtrate obtained was acidified to pH 3 by the addition of concentrated HCl aq. and the precipitated solid was collected by filtration and dried *in vacuo* to afford 2-nitro-isophthalic acid **227** as a white solid (6.96 g, 25.0 %).

Mp 316.0-317.6 °C, Lit.<sup>167</sup> 314.0-315.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3000-2500 (br, O-H), 1720 (C=O), 1605 (Ph), 1568, 1379 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{CO}, 200 \text{ MHz})$  8.32 (2H, d, *J* 7.7, PhH), 7.72 (1H, t, *J* 7.7, PhH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{CO}, 63 \text{ MHz})$  162.5 (C), 134.1, 129.9 (CH), 129.4, 123.7 (C); *m/z* (ES<sup>-</sup>) 210 (M-H)<sup>-</sup>.

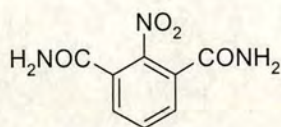
### 2-Nitro-isophthaloyl dichloride **228**.<sup>168</sup>



Phosphorus pentachloride (28.00 g, 0.1 mmol), phosphorus oxychloride (18.42 g, 11.2 mL) and 2-nitro-isophthalic acid **227** (5.63 g, 26.7 mmol) were heated under reflux for 10 h whereafter the liquid was slowly poured into a stirred ice mixture to hydrolyse any unreacted phosphorus pentachloride. The yellow solid was collected by filtration, washed several times with water, dried *in vacuo* and recrystallised (hexane) to afford 2-nitro-isophthaloyl dichloride **228** as orange crystals (6.34 g, 95.8 %).

Mp 128.5-131.0 °C, Lit.<sup>168</sup> 129.0-131.5 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3095 (CH), 1770 (C=O), 1590 (Ph), 1556, 1362 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{CO}, 200 \text{ MHz})$  8.33 (2H, d, *J* 7.9, PhH), 8.06 (1H, t, *J* 7.9, PhH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{CO}, 63 \text{ MHz})$  204.6, 137.2, 136.5 (C), 135.8, 130.9 (CH); *m/z* (CI<sup>+</sup>) 249 (MH<sup>+</sup>), 218 (M-NO), 202 (M-NO<sub>2</sub>).

### 2-Nitro-isophthalamide **229**.<sup>165</sup>



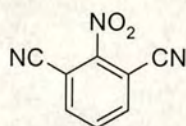
2-Nitro-isophthaloyl dichloride **228** (15.00 g, 60.0 mmol) was introduced slowly into a cooled (0 °C) beaker of concentrated ammonia solution (30 mL) and the resulting



heterogeneous mixture stirred at 25 °C for 30 min. The diamide was collected by gravity filtration, dried *in vacuo* and the resulting solid purified by recrystallisation (water) to afford *2-nitro-isophthalamide* **229** as cream needle-like crystals (3.18 g, 66.6 %).

Mp 292.2-293.6 °C, Lit.<sup>169</sup> 288.0-290.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3406, 3249 (NH<sub>2</sub>), 1664 (C=O, NH), 1613 (Ph), 1531, 1360 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{SO}, 200 \text{ MHz})$  8.29 (4H, br s, CONH<sub>2</sub>), 7.73 (2H, d, *J* 9.5, PhH), 7.66 (1H, t, *J* 9.5, PhH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{SO}, 63 \text{ MHz})$  166.3, 147.1 (C), 131.2 (CH), 131.1 (C), 130.1 (CH); *m/z* (CI<sup>+</sup>) 210 (MH<sup>+</sup>), 192 (M-NH<sub>3</sub>).

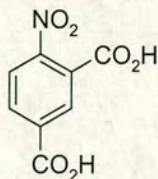
### 2-Nitro-isophthalonitrile **230**.<sup>170</sup>



A mixture of *2-nitro-isophthalamide* **229** (3.00 g, 14.4 mmol) and phosphorus pentoxide (58.60 g, 412.7 mmol) was heated *in vacuo* at 160 °C for 1.5 h. Soxhlet extraction of the powdered reaction mixture followed by concentration of the extract to *ca.* 20 mL and addition of petrol afforded a precipitate. The solid was removed by filtration and dried *in vacuo* to yield *2-nitro-isophthalonitrile* **230** as yellow crystals (0.91 g, 36.7 %).

Mp 151.0-152.3 °C, Lit.<sup>170</sup> 152.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3098 (CH), 2243 (CN), 1612 (Ph), 1541, 1345 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{SO}, 200 \text{ MHz})$  8.48 (2H, d, *J* 7.6, PhH), 8.11 (1H, t, *J* 7.6, PhH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{SO}, 63 \text{ MHz})$  150.1 (C), 139.6, 134.8 (CH), 114.5, 109.2 (C); *m/z* (EI) 173 (M<sup>+</sup>), 143 (M-NO), 127 (M-NO<sub>2</sub>), 100 (M-NO<sub>2</sub>-HCN); GC: R<sub>t</sub> 4.4 min, oven temp 150 °C.

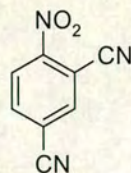


**4-Nitro-isophthalic acid 232.**<sup>171</sup>

3-Methyl-4-nitro-benzoic acid **231** (20.00 g, 10.1 mmol), distilled water (400 mL) and potassium permanganate (40.20 g, 2.5 eq.) were placed in a 3-necked flask fitted with a thermometer, a magnetic stirrer and a reflux condenser. The resulting mixture was stirred vigorously for 5 min, then heated to 85 °C after which the internal temperature was maintained at 80 °C using an ice bath to control the initial exothermic reaction. Upon completion the solution was heated under reflux for 2 h at 90 °C, cooled to 75 °C and filtered through a bed of Celite<sup>TM</sup>. The yellow filtrate obtained was acidified to pH 3 by the addition of concentrated HCl aq. and filtered to remove unreacted starting material. Concentration of the filtrate under reduced pressure afforded a solid which contained a mixture of both the starting material and the product. Purification by flash-column chromatography (ethyl acetate:chloroform, 1:1) afforded *4-nitro-isophthalic acid 232* as a cream solid (2.53 g, 10.5 %).

$R_f$  (EtOAc:CHCl<sub>3</sub>, 1:1) 0.14; Mp 243.5-244.8 °C, Lit.<sup>172</sup> 245.0-246.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3150 (br, O-H), 1699 (C=O), 1609 (Ph), 1539, 1380 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 12.2 (2H, br, COOH), 8.29 (1H, d,  $J$  1.8, PhH), 8.08 (1H, dd,  $J$  8.3 and 1.8, PhH), 7.84 (1H, d,  $J$  8.3, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 166.0, 165.6, 151.3, 134.8, 131.8 (C), 131.4, 131.0, 123.3 (CH);  $m/z$  (ES<sup>-</sup>) 210 (M-H)<sup>-</sup>, 181 (M-NO), 165 (M-NO<sub>2</sub>).



**4-Nitro-isophthalonitrile 233.**<sup>171</sup>

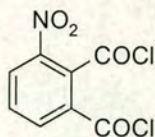
A mixture of 4-nitro-isophthalic acid **232** (2.03 g, 9.6 mmol), *para*-toluenesulphonamide (3.50 g, 20.7 mmol) and phosphorus pentachloride (8.60 g, 14.3 mmol) was heated at 200 °C for 30 min with stirring. Upon cooling, chloroform (20 mL) was added and the resulting solution was heated under reflux for 30 min, after which time 10 % NaHCO<sub>3</sub> (w/v) was added until a stable pH was obtained and the effervescence had stopped. The organic layer was separated, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure to afford a brown oil. Purification by flash-column chromatography (ethyl acetate:chloroform, 1:1) afforded *4-nitro-isophthalonitrile* **233** as a light brown solid (0.36 g, 21.6 %).

R<sub>f</sub> (EtOAc:CHCl<sub>3</sub>, 1:1) 0.65; Mp 122.0-123.5 °C, Lit.<sup>173</sup> 124.0-125.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3102 (CH), 2249 (CN), 1580 (Ph), 1539, 1353 (NO<sub>2</sub>);  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 200 MHz) 8.47 (1H, d, *J* 8.6, PhH), 8.22 (1H, d, *J* 0.5, PhH), 8.16 (1H, dd, *J* 8.6 and 0.5, PhH);  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 63 MHz) 138.6, 137.0, 126.4 (CH), 188.7, 144.7, 112.8, 110.2, 1091.6 (C); *m/z* (EI) 173 (M<sup>+</sup>), 143 (M-NO), 127 (M-NO<sub>2</sub>), 100 (M-NO<sub>2</sub>-HCN); GC: R<sub>t</sub> 9.5 min, oven temp 150 °C.



### 6.3 Synthesis of dicyano(di)nitroarenes.

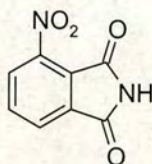
#### 3-Nitro-phthaloyl dichloride **235**.



Under an inert atmosphere, 3-nitro-phthalic acid **234** (2.00 g, 9.5 mmol, pre-dried at 100 °C for 1 h) was heated under reflux with thionyl chloride (2.5 eq.) for *ca.* 24 h. Excess thionyl chloride was removed *in vacuo* to yield an oil which afforded on cooling pale yellow needle-like crystals of 3-nitro-phthaloyl dichloride **235** (1.84 g, 78.0 %).

Mp 137.1-137.9 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3021 (CH), 1773 (C=O), 1615 (Ph), 1524, 1339 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.78 (2H, m, PhH), 7.47 (1H, dd, *J* 8.1 and 8.0, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 173.3, 168.6, 148.1, 135.4 (C), 132.9 (CH), 130.2 (C), 128.8, 122.3 (CH); *m/z* (EI) 248 (M<sup>+</sup>), 218 (M-NO).

#### 4-Nitro-isoindole-1,3-dione **236**.



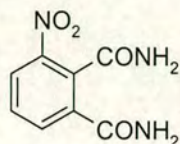
3-Nitro-phthaloyl dichloride **235** (1.00 g, 4.0 mmol) was introduced slowly into a cooled (0 °C) beaker of concentrated ammonia solution (5 mL) and the resulting heterogeneous mixture stirred at room temperature for 24 h. The ammonia solution was concentrated and dried *in vacuo* to afford 4-nitro-isoindole-1,3-dione **236** as a pale yellow solid (564.0 mg, 73.4 %).

Mp 214.2-215.9 °C, Lit.<sup>174</sup> 215.0-216.0 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3082 (CH), 1780, 1730 (C=O), 1620 (Ph), 1542, 1362 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 11.4 (1H, br s, OCNHCO), 8.24 (1H, dd, *J* 7.7 and 0.9, PhH), 8.05 (2H, m, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO,



63 MHz) 167.4, 164.8, 144.5 (C), 136.2 (CH), 134.8 (C), 128.3, 126.9 (CH), 124.0 (C);  $m/z$  (EI) 192 ( $M^+$ ), 146 (M-NO<sub>2</sub>).

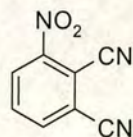
### 3-Nitro-phthalamide **237**.<sup>175</sup>



Concentrated ammonia solution (2 mL) was added to pulverised 4-nitro-isoindole-1,3-dione **236** (500.0 mg, 2.6 mmol) to form a pale brown suspension. The stirred reaction mixture was heated at 45 °C for 5 h, after which time the solid was removed by filtration, washed with cold water and dried to a constant weight affording 3-nitro-phthalamide **237** as a light yellow solid (337.5 mg, 62.1 %).

Mp 198.2-199.8 °C, Lit.<sup>176</sup> 200.0-201.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3341, 3125 (NH<sub>2</sub>), 3011 (CH), 1669 (C=O), 1621 (NH), 1599 (Ph), 1537, 1352 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.45 (2H, br s, CONH<sub>2</sub>), 8.19 (1H, d,  $J$  7.7, PhH), 8.13 (1H, br s, CONH<sub>2</sub>), 7.95 (1H, d,  $J$  7.7, PhH), 7.58 (1H, br s, CONH<sub>2</sub>), 6.68 (1H, dd,  $J$  8.0, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 167.5, 164.1, 146.2, 136.5 (C), 131.7, 130.6 (CH), 128.3 (C), 123.4 (CH);  $m/z$  (ES<sup>+</sup>) 210 (MH<sup>+</sup>), 164 (M-NO<sub>2</sub>), 120 (M-NO<sub>2</sub>-CONH<sub>2</sub>).

### 3-Nitro-phthalonitrile **238**.<sup>175</sup>



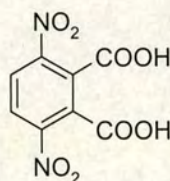
DMF (1.8 mL) was cooled to 5 °C under a nitrogen atmosphere and thionyl chloride (4.79 g, 7.8 mL) was added drop-wise. The solution was stirred for 3 h, after which time 3-nitro-phthalamide **237** (300.0 mg, 1.7 mmol) was added at such a rate as to keep the temperature below 10 °C. The reaction was stirred for 6 h at 0-5 °C then brought to room temperature and stirred overnight. The solution was poured onto ice and the crystallised product collected by vacuum filtration and washed several times



with cold water. The product was dried *in vacuo* to afford 3-nitro-phthalonitrile **238** as a pale yellow solid (210.3 mg, 71.5 %).

Mp 159.3-161.8 °C, Lit.<sup>175</sup> 160.0-162.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3074 (CH), 2249 (CN), 1594 (Ph), 1540, 1360 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{SO}, 200 \text{ MHz})$  8.72 (1H, dd, *J* 8.5, PhH), 8.62 (1H, dd, *J* 8.5, PhH), 8.25 (1H, dd, *J* 8.5, PhH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{SO}, 63 \text{ MHz})$  147.1 (C), 137.2, 134.9, 128.7 (CH), 116.3, 112.8, 113.2, 110.1 (C); *m/z* (EI) 173 (M<sup>+</sup>), 143 (M-NO), 127(M-NO<sub>2</sub>), 73 (M-NO<sub>2</sub>-2HCN).

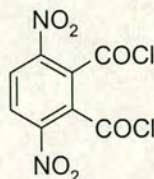
### 3,6-Dinitro-phthalic acid **240**.



To a solution of 3,6-dinitro-phthalic acid mono-pyridinium salt **239** (100.0 mg, 0.3 mmol) in the minimum amount of deionised water was added acidic resin (1.25 g, 6.0 mmol) and the mixture stirred for 2 h. The solution was pH tested to confirm the formation of acid. The resin was removed by filtration and further washed with deionised water. The filtrate and washings obtained were concentrated, dried *in vacuo* to afford 3,6-dinitro-phthalic acid **240** as a pale yellow solid (82.3 mg, 91.5 %)

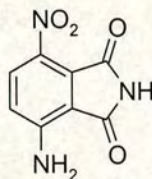
Mp 199.9-201.5 °C, Lit.<sup>177</sup> 201.0-202.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3284 (br, O-H), 1722 (C=O), 1622 (Ph), 1552, 1346 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{SO}, 200 \text{ MHz})$  8.37 (2H, s, PhH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{SO}, 63 \text{ MHz})$  165.4, 151.5, 132.4 (C), 129.5 (CH); *m/z* (ES<sup>-</sup>) 254 (M-2H)<sup>2-</sup>, 210 (M-NO<sub>2</sub>), 165 (M-NO<sub>2</sub>).



**3,6-Dinitro-phthaloyl dichloride 241.**

Under an inert atmosphere, 3,6-dinitro-phthalic acid **240** (75.0 mg, 0.3 mmol, pre-dried at 100 °C for 1 h) was heated under reflux with thionyl chloride (2.5 eq.) for *ca.* 24 h. Excess thionyl chloride was removed *in vacuo* to yield an oil which afforded on cooling 3,6-dinitro-phthaloyl dichloride **241** as a pale brown solid (66.3 mg, 75.4 %).

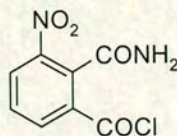
Mp > 300.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  2923 (CH), 1785 (C=O), 1595 (Ph), 1535, 1356 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.91 (2H, s, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 163.2, 150.1, 132.9 (C), 129.6 (CH);  $m/z$  (EI) 293 (M<sup>+</sup>), 247 (M-NO<sub>2</sub>), Found (M<sup>+</sup>) 291.9183, C<sub>8</sub>H<sub>2</sub>N<sub>2</sub>O<sub>6</sub>Cl<sub>2</sub> requires 291.9290.

**4-Amino-7-nitro-isoindole-1,3-dione 242.**

3,6-Dinitro-phthaloyl dichloride **241** (60.0 mg, 0.2 mmol) was introduced slowly into a cooled (0 °C) beaker of concentrated ammonia solution (2 mL) and the resulting heterogeneous mixture stirred at 25 °C for 24 h. The diamide was collected by gravity filtration, dried *in vacuo* to afford 4-amino-7-nitro-isoindole-1,3-dione **242** as a pale brown solid (30.1 mg, 72.7 %).

Mp 206.0-208.3 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3487, 3215 (NH<sub>2</sub>), 2954 (CH), 1726, 1718 (C=O), 1622 (Ph), 1549, 1367 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 12.0 (1H, br s, OCNHCO), 8.23 (1H, d, *J* 8.6, PhH), 7.30 (1H, d, *J* 8.6, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 165.2, 163.5, 148.2, 142.6, 131.2 (C), 130.1 (CH), 121.9 (C), 120.1 (CH);  $m/z$  (EI) 207 (M<sup>+</sup>), 161 (M-NO<sub>2</sub>), 134 (M-NO<sub>2</sub>-NO).



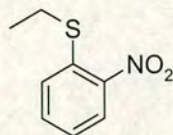
**2-Carbamoyl-3-nitro-benzoyl chloride 246.**

Under a nitrogen atmosphere, 3-nitro-phthaloyl dichloride **235** (500.0 mg, 2.0 mmol) in anhydrous dichloromethane (5 mL) was added to an ice-cold solution of hexamethyldisilazide (976.0 mg, 1.3 mL) in anhydrous dichloromethane (15 mL) and left to stir overnight at room temperature. Methanol (2 mL) was added and the solution washed with 5 % aqueous sulfuric acid (2 x 20 mL) and a saturated solution of ammonium acetate (2 x 20 mL). The organic extract was dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure to afford *2-carbamoyl-3-nitro-benzoyl chloride 246* as a white solid (304.1 mg, 66.4 %).

Mp 200.5-202.0 °C;  $\nu_{\text{max}}$ (KBr)/cm<sup>-1</sup> 6005 (CH), 1650, 1635 (C=O, NH), 1612 (Ph), 1552, 1329 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.61 (1H, dd, *J* 7.8 and 1.2, PhH), 8.52 (1H, dd, *J* 8.0 and 1.2, PhH), 7.99 (1H, dd, *J* 8.0 and 7.8, PhH), 7.21 (2H, br s, NH<sub>2</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 166.6, 166.1, 147.5 (C), 134.4 (CH), 133.1 (C), 132.9 (CH), 131.8 (C), 127.2 (CH); *m/z* (EI) 229 (M<sup>+</sup>), 183 (M-NO<sub>2</sub>), 139 (M-NO<sub>2</sub>-CONH<sub>2</sub>), Found (M<sup>+</sup>) 227.9902, C<sub>8</sub>H<sub>5</sub>N<sub>2</sub>O<sub>4</sub>Cl requires 227.9938.

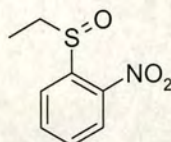


## 6.4 Synthesis of nitroarene-sulfinyls.

1-Ethylsulfanyl-2-nitrobenzene **251**.<sup>178</sup>

Under an inert atmosphere, ethanethiol (5.87 g, 7 mL) was added to ethanol (280 mL) and stirred for 5 min after which time a solution of NaOH (3.80 g, 95.0 mmol) in water (28 mL) was added. This solution was added to a stirred solution of *ortho*-chloro-2-nitrobenzene **250** (15.00 g, 94.9 mmol) in ethanol (95 mL), the mixture heated under reflux for 10 min and concentrated under reduced pressure to afford a red oil. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) to afford *1-ethylsulfanyl-2-nitrobenzene* **251** as a yellow oil (15.82 g, 73.0 %).

$R_f$  (EtOAc) 0.70; Bp 147.2 °C, Lit.<sup>179</sup> 149.0-150.0 °C  $\nu_{\max}(\text{film})/\text{cm}^{-1}$  2972 (CH), 1592 (Ph), 1512, 1337 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.16 (1H, dd,  $J$  8.4 and 1.5, PhH), 7.53 (1H, td,  $J$  8.4, 7.0 and 1.5, PhH), 7.39 (1H, dd,  $J$  7.0 and 1.5, PhH), 7.22 (1H, td,  $J$  8.4, 7.0 and 1.5, PhH), 2.97 (2H, q,  $J$  7.3, CH<sub>2</sub>), 1.38 (3H, t,  $J$  7.3, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 137.7 (C), 133.3 (CH), 131.7 (C), 126.3, 125.9, 124.1, 26.1 (CH<sub>2</sub>), 12.6 (CH<sub>3</sub>);  $m/z$  (EI) 183 (M<sup>+</sup>), 154 (M-C<sub>2</sub>H<sub>5</sub>), 122 (M-SC<sub>2</sub>H<sub>5</sub>), 92 (M-SC<sub>2</sub>H<sub>5</sub>-NO).

1-Ethanesulfinyl-2-nitrobenzene **261**.<sup>141</sup>

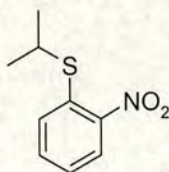
To a stirred solution of ethylsulfanyl-2-nitrobenzene **251** (4.00 g, 17.5 mmol) in chloroform (82 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (3.46 g, 20.0 mmol) in chloroform (40 mL). The reaction was stirred for 15 min then



left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq.  $\text{NaHCO}_3$  (50 mL) solution. The organic layer was separated, dried ( $\text{MgSO}_4$ ), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate) afforded *1-ethanesulfinyl-2-nitrobenzene* **261** as a yellow solid (3.45 g, 99.1 %).

$R_f$  (EtOAc) 0.39; Mp 95.2 °C, Lit.<sup>180</sup> 96.0-99.0 °C;  $\nu_{\text{max}}$ (KBr)/ $\text{cm}^{-1}$  3097 (CH), 1596 (Ph), 1521, 1346 ( $\text{NO}_2$ ), 1067 (S=O);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 8.27 (2H, m, PhH), 7.94 (1H, td,  $J$  7.7, 7.3 and 1.1, PhH), 7.71 (1H, td,  $J$  8.4, 7.7 and 1.5, PhH), 3.71 (1H, q,  $J$  7.3,  $\text{CH}_2$ ), 2.82 (1H, q,  $J$  10.3,  $\text{CH}_2$ ), 1.40 (1H, t,  $J$  10.3 and 7.3,  $\text{CH}_3$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 144.7, 142.8 (C), 135.0, 131.2, 127.1, 125.1 (CH), 49.5 ( $\text{CH}_2$ ), 6.71 ( $\text{CH}_3$ );  $m/z$  (EI) 199 ( $\text{M}^+$ ), 170 ( $\text{M}-\text{C}_2\text{H}_5$ ), 122 ( $\text{M}-\text{SOC}_2\text{H}_5$ ); HPLC (hexane:EtOH, 90:10):  $R_t$  17.3 and 19.8 min.

### **1-iso-Propylsulfonyl-2-nitrobenzene 252.**<sup>181</sup>



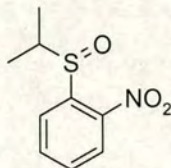
To a stirred solution of propane-2-thiol (18.04 g, 22 mL) in *iso*-propanol (degassed, 250 mL) was added a solution of NaOH (8.50 g, 212.5 mmol) in water (65 mL). The alcoholic solution of sodium mercaptide was added to a solution of *ortho*-chloro-2-nitrobenzene **250** (4.00 g, 25.3 mmol) in *iso*-propanol (degassed, 20 mL) and the reaction stirred at 40 °C for 5 h. After complete substrate consumption (TLC), the reaction mixture was poured into dilute HCl (2 %, 300 mL) and extracted with ether (3 x 200 mL). The combined organic extracts were dried ( $\text{MgSO}_4$ ), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *1-iso-propylsulfonyl-2-nitrobenzene* **252** as an orange/red oil (4.21 g, 84.4 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.59; Bp 102.0-103.5 °C, Lit.<sup>182</sup> 103.0-105.0 °C;  $\nu_{\text{max}}$ (film)/ $\text{cm}^{-1}$  3027 (CH), 1601 (Ph), 1565, 1354 ( $\text{NO}_2$ );  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 8.08 (1H, dd,  $J$  8.2 and 1.4, PhH), 7.50 (2H, m, PhH), 7.22 (1H, td,  $J$  8.2, 6.8 and 1.5,



PhH), 3.55 (1H, sept,  $J$  6.6, CH), 1.29 (6H, d,  $J$  6.6, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 147.1, 136.1 (C), 132.9, 128.2, 125.6, 124.6, 35.6 (CH), 22.2 (CH<sub>3</sub>);  $m/z$  (EI) 197 (M<sup>+</sup>), 154 (M-C<sub>3</sub>H<sub>7</sub>), 122 (M-SC<sub>3</sub>H<sub>7</sub>).

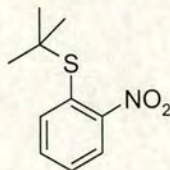
### 1-Nitro-2-(propane-2-sulfinyl)-benzene **262**.



To a stirred solution of sulfide **252** (4.00 g, 20.3 mmol) in chloroform (80 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (3.98 g, 23.0 mmol) in chloroform (50 mL). The reaction was stirred for 15 min then left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq. NaHCO<sub>3</sub> (50 mL) solution. The organic layer was separated, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *1-nitro-2-(propane-2-sulfinyl)-benzene* **262** as a yellow solid (1.40 g, 32.3 %).

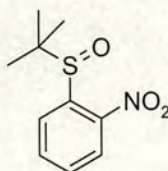
$R_f$  (EtOAc:Petrol, 1:1) 0.12; Mp 90.1-91.6 °C, Lit.<sup>183</sup> 89.0-91.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 2988 (CH), 1564 (Ph), 1530, 1321 (NO<sub>2</sub>), 1048 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.31 (1H, dd,  $J$  8.1 and 1.2, PhH), 8.16 (1H, dd,  $J$  7.9 and 1.4, PhH), 7.92 (1H, td,  $J$  7.9, 7.3 and 1.2, PhH), 7.67 (1H, td,  $J$  8.1, 7.3 and 1.4, PhH), 3.20 (1H, sept,  $J$  7.0 and 6.9, CH), 1.55 (3H, d,  $J$  7.0, CH<sub>3</sub>), 0.95 (3H, d,  $J$  6.9, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 144.7, 142.1 (C), 134.5, 131.0, 127.6, 125.2, 52.7 (CH), 18.8, 11.8 (CH<sub>3</sub>);  $m/z$  (EI) 213 (M<sup>+</sup>), 138 (M-SC<sub>3</sub>H<sub>7</sub>), 108 (M-SC<sub>3</sub>H<sub>7</sub>-NO); HPLC (hexane:EtOH, 99:1):  $R_t$  32.2 and 41.9 min.



**1-*tert*-Butylsulfanyl-2-nitrobenzene 253.**<sup>184</sup>

To a stirred solution of 2-methyl-propane-2-thiol sodium salt (1.17 g, 10.4 mmol) in *iso*-propanol (degassed, 250 mL) was added a solution of *ortho*-chloro-2-nitrobenzene **250** (1.40 g, 6.9 mmol) in *iso*-propanol (10 mL) and the reaction stirred at 40 °C for 5 h. After complete substrate consumption (TLC), the reaction mixture was poured into dilute HCl (2 %, 300 mL) and extracted with ether (3 x 200 mL). The combined organic extracts were dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ether:toluene, 8:2) afforded 1-*tert*-butylsulfanyl-2-nitrobenzene **253** as an oil (615.0 mg, 42.2 %).

$R_f$  (ether:toluene, 8:2) 0.17;  $\nu_{\max}$ (film)/cm<sup>-1</sup> 2911 (CH), 1601 (Ph), 1545, 1330 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.91 (1H, dd,  $J$  7.9 and 1.3, PhH), 7.52 (2H, m, PhH), 7.44 (1H, td,  $J$  7.9, 7.7 and 1.2, PhH), 1.30 (9H, s, C(CH<sub>3</sub>)<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 152.9 (C), 136.2, 132.1, 127.5 (CH), 125.2 (C), 120.9 (CH), 45.1 (C), 35.8 (CH<sub>3</sub>);  $m/z$  (EI) 211 (M<sup>+</sup>), 154 (M-C(CH<sub>3</sub>)<sub>3</sub>), 122 (M-SC(CH<sub>3</sub>)<sub>3</sub>), 92 (M-SC(CH<sub>3</sub>)<sub>3</sub>-NO).

**1-(2-Methyl-propane-2-sulfinyl)-2-nitrobenzene 263.**

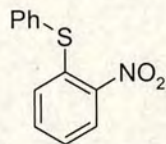
To a stirred solution of sulfide **253** (506.4 mg, 2.4 mmol) in chloroform (15 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (484.8 mg, 2.8 mmol) in chloroform (40 mL). The reaction was stirred for 15 min then left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq. NaHCO<sub>3</sub> (50 mL) solution. The organic layer was separated, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column



chromatography (ethyl acetate:petrol, 1:1) afforded *1-(2-methyl-propane-2-sulfinyl)-2-nitrobenzene* **263** as a yellow solid (276 mg, 50.7 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.24; Mp 79.2-80.9 °C, (Found C, 52.87; H, 5.91; N, 6.14;  $C_{10}H_{13}NSO_3$  requires C, 52.85; H, 5.77; N, 6.17);  $\nu_{max}(KBr)/cm^{-1}$  2985 (CH), 1590 (Ph), 1550, 1334 (NO<sub>2</sub>), 1055 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.34 (1H, dd,  $J$  7.7 and 1.2, PhH), 8.13 (1H, dd,  $J$  7.9 and 1.2, PhH), 8.01 (1H, td,  $J$  7.9, 7.8 and 1.2, PhH), 7.82 (1H, td,  $J$  7.8, 7.7 and 1.2, PhH), 1.40 (3H, s, CH<sub>3</sub>), 1.32 (3H, s, CH<sub>3</sub>), 1.29 (3H, s, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 150.1, 145.2 (C), 135.6, 130.2, 128.6, 125.7 (CH), 55.1 (C), 27.4 (CH<sub>3</sub>);  $m/z$  (EI) 227 (M<sup>+</sup>), 170 (M-C(CH<sub>3</sub>)<sub>3</sub>), 124 (M-C(CH<sub>3</sub>)<sub>3</sub>-NO<sub>2</sub>), Found (M<sup>+</sup>) 227.1264,  $C_{10}H_{13}NSO_3$  requires 227.0616; HPLC (hexane:EtOH, 99:1):  $R_t$  40.1 and 46.2 min.

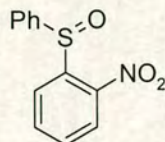
### 1-Nitro-2-phenylsulfanyl-benzene **254**.<sup>185</sup>



To a stirred solution of benzenethiol (11.05 g, 10.3 mL), NaHCO<sub>3</sub> (13.00 g, 156.6 mmol) in water (40 mL) was added a solution of *ortho*-chloro-2-nitrobenzene **250** (15.70 g, 99.4 mmol) in hot ethanol (33 mL) and the reaction heated at 65 °C. After 4 h, the solution was poured into water (175 mL) and stirred for 30 min at room temperature. The product was obtained by gravity filtration, washed several times with water and dried *in vacuo*. Recrystallisation (ethanol) afforded *1-nitro-2-phenylsulfanyl-benzene* **254** as yellow crystals (2.51 g, 10.9 %).

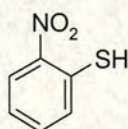
$R_f$  (EtOAc:Petrol, 3:2) 0.60; Mp 76.9-78.1 °C, Lit.<sup>186</sup> 78.0-81.0 °C;  $\nu_{max}(KBr)/cm^{-1}$  3064 (CH), 1590 (Ph), 1502, 1334 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.21 (1H, dd,  $J$  8.1 and 1.4, PhH), 7.58 (5H, m, Ph'H), 7.31 (2H, m, PhH), 6.84 (1H, dd,  $J$  8.2 and 1.4, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 144.8, 139.4 (C), 135.8, 133.3 (CH), 130.8 (C), 130.0, 129.9, 128.1, 125.6, 124.8 (CH);  $m/z$  (EI) 231 (M<sup>+</sup>), 122 (M-SC<sub>6</sub>H<sub>5</sub>), 76 (M-SC<sub>6</sub>H<sub>5</sub>-NO<sub>2</sub>).



**1-Benzenesulfinyl-2-nitrobenzene 264.**

To a stirred solution of sulfide **254** (2.01 g, 8.7 mmol) in chloroform (40 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (1.56 g, 9.0 mmol) in chloroform (20 mL). The reaction was stirred for 15 min then left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq. NaHCO<sub>3</sub> (50 mL) solution. The organic layer was separated, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 3:2) afforded *1-benzenesulfinyl-2-nitrobenzene 264* as a yellow solid (1.34 g, 62.7 %).

R<sub>f</sub> (EtOAc:Petrol, 3:2) 0.36; Mp 90.3-91.7 °C, Lit.<sup>187</sup> 92.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3083 (CH), 1592 (Ph), 1525, 1344 (NO<sub>2</sub>), 1064 (S=O);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.58 (1H, dd, *J* 7.8 and 1.4, PhH), 8.25 (1H, dd, *J* 8.1 and 1.2, PhH), 8.02 (2H, m, Ph'H), 7.71 (2H, m, PhH), 7.34 (3H, m, Ph'H);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 145.0, 144.4, 143.7 (C), 135.3, 131.4, 131.3, 129.1, 126.6, 126.2 and 125.2 (CH); *m/z* (EI) 247 (M<sup>+</sup>), 170 (M-C<sub>6</sub>H<sub>5</sub>), 138 (M-SC<sub>6</sub>H<sub>5</sub>); HPLC (hexane:*iso*-propanol, 95:5): R<sub>t</sub> 48.9 and 68.9 min.

***Ortho*-nitro-benzenethiol 256.**<sup>188, 189</sup>

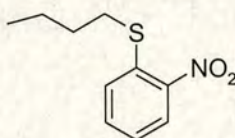
*Ortho*-nitro-benzenesulfonyl chloride **255** (2.55 g, 11.5 mmol) was added with stirring to a mixture of crushed ice (10.00 g) and concentrated sulfuric acid (55 mL). Zinc dust (1.79 g, 27.6 mmol) was added in portions such that the temperature did not rise above 10 °C. The mixture was concentrated under reduced pressure and the residue was extracted with ether (2 x 40 mL). The combined organic extracts were dried (MgSO<sub>4</sub>), filtered and concentrated *in vacuo*. The crude product was purified



by flash-column chromatography (ethyl acetate:petrol, 3:2) to afford *ortho*-nitro-benzenethiol **256** as a pale yellow solid (1.57 g, 88.2 %).

$R_f$  (EtOAc:Petrol, 3:2) 0.30; Mp 55.1-56.3 °C, Lit.<sup>190</sup> 55.0-56.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3026 (CH), 1613 (Ph), 1526, 1341 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.09 (1H, dd,  $J$  8.0 and 1.5, PhH), 7.52 (2H, m, PhH), 7.41 (1H, td,  $J$  8.0, 7.8 and 1.4, PhH), 4.00 (1H, br s, SH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 144.2 (C), 133.1, 131.9 (CH), 131.0 (C), 124.8, 124.2 (CH);  $m/z$  (EI) 155 (M<sup>+</sup>), 109 (M-NO<sub>2</sub>).

### 1-Butylsulfanyl-2-nitrobenzene **257**.<sup>191</sup>



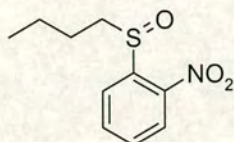
A solution of *ortho*-nitro-benzenethiol **256** (1.55 g, 10.0 mmol) in ethanol (25 mL) was placed in a 3-necked flask equipped with a magnetic stirrer, an addition funnel, a reflux condenser and a nitrogen inlet. Potassium hydroxide (1.10 g, 20.0 mmol) was added to the mixture while heating to 60 °C. After 30 min, the solution was cooled to room temperature and a solution of bromo-butane (1.37 g, 1.1 mL) in ethanol (5 mL) was added drop-wise. Upon complete addition, the mixture was heated under reflux for 1 h, cooled, diluted with water (30 mL) and extracted with ether (3 x 40 mL). The combined organic extracts were dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. The crude sulfide was purified by flash-column chromatography (ether:hexane, 1:1) to afford *1-butylsulfanyl-2-nitrobenzene* **257** as an oil (980.0 mg, 46.4 %).

$R_f$  (ether:hexane, 1:1) 0.48; Bp 136.0-137.5 °C, Lit.<sup>192</sup> 137.0-138.0 °C  $\nu_{\max}(\text{film})/\text{cm}^{-1}$  2972 (CH), 1603 (Ph), 1510, 1334 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.19 (1H, dd,  $J$  8.2 and 1.3, PhH), 7.65 (1H, td,  $J$  8.1, 1.3, PhH), 7.38 (1H, dd,  $J$  8.1 and 1.3, PhH), 7.15 (1H, td,  $J$  8.2, 8.1 and 1.3, PhH), 2.90 (2H, t,  $J$  4.9, SCH<sub>2</sub>), 1.52 (4H, m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 0.92 (3H, t,  $J$  4.9 CH<sub>3</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 144.1 (C), 131.2



(CH), 130.1 (C), 125.3, 124.9, 124.2 (CH), 36.2 (SCH<sub>2</sub>), 34.1 (CH<sub>2</sub>Et), 21.3 (CH<sub>2</sub>), 13.1 (CH<sub>3</sub>); *m/z* (EI) 211 (M<sup>+</sup>), 122 (M-SC<sub>4</sub>H<sub>9</sub>), 76 (M-SC<sub>4</sub>H<sub>9</sub>-NO<sub>2</sub>).

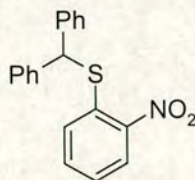
**1-(Butane-1-sulfinyl)-2-nitrobenzene 265.**



To a stirred solution of sulfide **257** (760.0 mg, 3.6 mmol) in chloroform (20 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (690.0 mg, 4.0 mmol) in chloroform (10 mL). The reaction was stirred for 15 min then left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq. NaHCO<sub>3</sub> (50 mL) solution. The organic layer was separated, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *1-(butane-1-sulfinyl)-2-nitrobenzene 265* as a yellow solid (521.0 mg, 63.8 %).

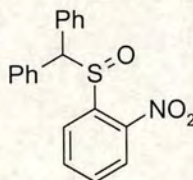
*R<sub>f</sub>* (EtOAc:Petrol, 1:1) 0.31; *Mp* 51.2 °C, Lit.<sup>191</sup> 50.0-52.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3103 (CH), 1580 (Ph), 1525, 1342 (NO<sub>2</sub>) 1031 (S=O);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.70 (1H, dd, *J* 8.1 and 1.2, PhH), 8.28 (1H, dd, *J* 7.9 and 1.1, PhH), 7.79 (1H, td, *J* 8.1, 7.8 and 1.1, PhH), 7.32 (1H, td, *J* 7.9, 7.8 and 1.2, PhH), 3.11 (2H, m, S(O)CH<sub>2</sub>), 1.80 (4H, m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>), 0.96 (3H, t, *J* 5.8, CH<sub>3</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 147.2, 145.1 (C), 138.4, 133.1, 132.4, 127.5 (CH), 55.7 (SCH<sub>2</sub>), 48.1 (CH<sub>2</sub>Et), 38.1 (CH<sub>2</sub>), 10.3(CH<sub>3</sub>); *m/z* (EI) 227 (M<sup>+</sup>), 170 (M-C<sub>4</sub>H<sub>9</sub>), 138 (M-SC<sub>4</sub>H<sub>9</sub>O); HPLC (hexane:EtOH, 90:10): *R<sub>t</sub>* 23.4 and 27.6 min.



**1-Benzhydrylsulfanyl-2-nitrobenzene 259.**<sup>193</sup>

To a pre-cooled solution of diphenylmethane **258** (4.44 g, 4.4 mL) and *ortho*-nitrobenzene-sulfenyl chloride **255** (5.00 g, 26.3 mmol) in anhydrous toluene (26 mL) under nitrogen was added drop-wise a solution of triethylamine (2.69 g, 3.7 mL) in anhydrous toluene (6 mL) and the reaction stirred for 6 h. The mixture was filtered and the filtrate concentrated under reduced pressure to yield an orange oil, which crystallised when treated with petrol. The crystals were collected under gravity filtration and recrystallised (toluene) to afford *1-benzhydrylsulfanyl-2-nitrobenzene 259* as pale orange crystals (874.0 mg, 10.3 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.58; Mp 157.9 °C, Lit.<sup>193</sup> 158.0-161.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3098 (CH), 1599 (Ph), 1528, 1353 ( $\text{NO}_2$ );  $\delta_{\text{H}}((\text{CD}_3)_2\text{SO}, 200 \text{ MHz})$  8.03 (1H, dd,  $J$  8.2 and 1.4, PhH), 7.80 (8H, m, Ph'H), 7.72 (2H, m, PhH), 7.51 (2H, m, Ph'H), 7.47 (1H, td,  $J$  8.2, 7.2 and 1.4, PhH), 4.85 (1H, s, CH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{SO}, 63 \text{ MHz})$  146.2, 145.2 (C), 135.0 (CH), 132.7 (C), 130.9, 128.3, 127.0, 126.9, 125.3, 124.8, 48.2 (CH);  $m/z$  (EI) 321 ( $\text{M}^+$ ), 244 ( $\text{M}-\text{C}_6\text{H}_5$ ), 154 ( $\text{M}-\text{CH}(\text{C}_6\text{H}_5)_2$ ), 122 ( $\text{M}-\text{SCH}(\text{C}_6\text{H}_5)_2$ ).

**1-(Diphenyl-methanesulfinyl)-2-nitrobenzene 266.**

To a stirred solution of sulfide **259** (898.8 mg, 2.8 mmol) in chloroform (15 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (554.1 mg, 3.2 mmol) in chloroform (10 mL). The reaction was stirred for 15 min then left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq.  $\text{NaHCO}_3$  (50 mL) solution. The organic layer was separated, dried ( $\text{MgSO}_4$ ), filtered

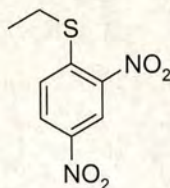


and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *1-(diphenyl-methanesulfinyl)-2-nitrobenzene* **266** as a pale orange solid (229.0 mg, 31.2 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.32; Mp 159.9 °C, Lit.<sup>193</sup> 152.0-154.2 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3078 (CH), 1584 (Ph), 1544, 1371 (NO<sub>2</sub>), 1087 (S=O);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.75 (1H, dd,  $J$  8.5 and 1.1, PhH), 8.32 (1H, dd,  $J$  8.25 and 1.3, PhH), 7.79 (8H, m, Ph'H), 7.62 (2H, m, PhH), 7.50 (2H, m, Ph'H), 5.42 (1H, s, CH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 148.6, 145.2, 142.1 (C), 138.4, 135.7, 130.2, 128.5, 127.9, 126.7, 125.1, 70.2 (CH);  $m/z$  (EI) 337 (M<sup>+</sup>), 138 (M-SCH(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub>), 108 (M-SCH(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub>-NO); HPLC (hexane:EtOH, 60:40):  $R_t$  29.1 and 36.4 min.

## 6.5 Synthesis of dinitroarenes-sulfinyls.

### 1-Ethylsulfanyl-2,4-dinitrobenzene **270**.<sup>178</sup>



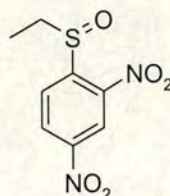
Under an inert atmosphere, ethanethiol (4.54 g, 5.4 mL) was added to ethanol (220 mL) and stirred for 5 min after which time a solution of NaOH (2.90 g, 72.5 mmol) in water (22 mL) was added. This solution was added to a stirred solution of *ortho*-chloro-2,4-dinitrobenzene **269** (15.00 g, 73.9 mmol) in ethanol (95 mL) and the reaction heated under reflux for 10 min and filtered quickly. The crystallised sulfide was collected by filtration and dried *in vacuo*, recrystallisation (ethanol) afforded *1-ethylsulfanyl-2,4-dinitrobenzene* **270** as yellow needle-like crystals (3.17 g, 19.2 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.65; Mp 113.2-114.6 °C, Lit.<sup>194</sup> 114.0-115.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3005 (CH), 1618 (Ph), 1531, 1350 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.50 (1H, d,  $J$  2.5, PhH), 8.35 (1H, dd,  $J$  9.0 and 2.5, PhH), 7.56 (1H, d,  $J$  9.0, PhH), 3.08 (2H, q,  $J$  7.4, CH<sub>2</sub>), 1.45 (3H, t,  $J$  7.4, CH<sub>3</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63



MHz) 147.2, 144.5, 143.5 (C), 126.9, 126.3, 121.6 (CH), 26.6 (CH<sub>2</sub>), 12.3 (CH<sub>3</sub>); *m/z* (EI) 228 (M<sup>+</sup>), 167 (M-SC<sub>2</sub>H<sub>5</sub>), 137 (M-SC<sub>2</sub>H<sub>5</sub>-NO), 121 (M-SC<sub>2</sub>H<sub>5</sub>-NO<sub>2</sub>); HPLC (hexane:EtOH, 80:20): R<sub>t</sub> 21.6 min.

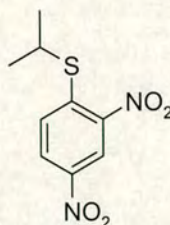
### 1-Ethane-sulfinyl-2,4-dinitrobenzene **275**.



To a stirred solution of sulfide **270** (2.01 g, 8.8 mmol) in chloroform (45 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (1.59 g, 9.2 mmol) in chloroform (25 mL). The reaction was stirred for 15 min then left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq. NaHCO<sub>3</sub> (50 mL) solution. The organic layer was separated, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *1-ethane-sulfinyl-2,4-dinitrobenzene* **275** as yellow crystals (1.36 g, 63.4 %).

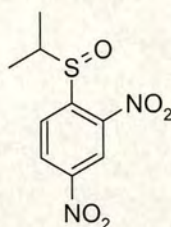
R<sub>f</sub> (EtOAc:Petrol, 1:1) 0.45; Mp 111.5 °C, Lit.<sup>195</sup> 112.0-113.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3082 (CH), 1602 (Ph), 1533, 1343 (NO<sub>2</sub>), 1035 (S=O);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.11 (1H, d, *J* 2.1, PhH), 8.73 (1H, dd, *J* 8.6 and 2.2, PhH), 8.49 (1H, d, *J* 8.6, PhH), 3.35 (1H, q, *J* 7.5, CH<sub>2</sub>), 2.89 (1H, q, *J* 7.5, CH<sub>2</sub>), 1.34 (3H, t, *J* 7.5, CH<sub>3</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 1501, 149.3, 144.9 (C), 129.2, 128.7, 120.4 (CH), 49.7 (CH<sub>2</sub>), 11.3 (CH<sub>3</sub>); *m/z* (EI) 244 (M<sup>+</sup>), 183 (M-SC<sub>2</sub>H<sub>5</sub>), 137 (M-SC<sub>2</sub>H<sub>5</sub>-NO<sub>2</sub>); HPLC (hexane:EtOH, 50:20): R<sub>t</sub> 34.7 and 55.5 min.



**1-iso-Propylsulfany-2,4-dinitrobenzene 271.**<sup>196</sup>

Under an inert atmosphere, 2-propanethiol (6.56 g, 8 mL) was added to ethanol (220 mL) and stirred for 5 min after which time a solution of NaOH (2.96 g, 74.0 mmol) in water (22 mL) was added. This solution was added to a stirred solution of *ortho*-chloro-2,4-dinitrobenzene **269** (15.00 g, 73.9 mmol) in ethanol (74.1 mL) and the reaction heated under reflux for 10 min and filtered quickly. The crystallised sulfide was collected by filtration and dried *in vacuo*, recrystallisation (ethanol) afforded *1-iso-propylsulfinyl-2,4-dinitrobenzene 271* as yellow crystals (10.99 g, 61.5 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.64; Mp 92.9-93.5 °C, Lit.<sup>194</sup> 93.0-94.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3097 (CH), 1584 (Ph), 1528, 1338 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.00 (1H, d,  $J$  2.4, PhH), 8.35 (1H, dd,  $J$  9.0 and 2.4, PhH), 7.60 (1H, d,  $J$  9.0, PhH), 3.66 (1H, q,  $J$  6.7, CH), 1.46 (6H, d,  $J$  6.7, CH<sub>3</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 146.4, 145.1, 143.5 (C), 127.4, 126.7, 121.7, 36.0 (CH), 22.0 (CH<sub>3</sub>);  $m/z$  (EI) 242 (M<sup>+</sup>), 199 (M-C<sub>3</sub>H<sub>7</sub>), 169 (M-C<sub>3</sub>H<sub>7</sub>-NO).

**1-(Propane-2-sulfinyl)-2,4-dinitrobenzene 276.**

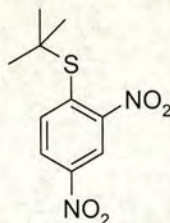
To a stirred solution of sulfide **271** (2.01 g, 8.3 mmol) in chloroform (40 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (1.49 g, 8.6 mmol) in chloroform (25 mL). The reaction was stirred for 15 min then left to stand overnight



at room temperature, whereafter it was filtered and washed twice with a sat. aq.  $\text{NaHCO}_3$  (50 mL) solution. The organic layer was separated, dried ( $\text{MgSO}_4$ ), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *1-(propane-2-sulfinyl)-2,4-dinitrobenzene* **276** as yellow crystals (450.0 mg, 21.1 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.48; Mp 100.0-102.2 °C, (Found C, 42.16; H, 4.06; N, 10.76;  $\text{C}_9\text{H}_{10}\text{SN}_2\text{SO}_5$  requires C, 41.86; H, 3.91; N, 10.85);  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3125 (CH), 1622 (Ph), 1531, 1340 ( $\text{NO}_2$ ), 1064 (S=O);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 9.11 (1H, d,  $J$  2.2, PhH), 8.71 (1H, dd,  $J$  8.6 and 2.2, PhH), 8.40 (1H, d,  $J$  8.6, PhH), 3.29 (1H, q,  $J$  7.0, CH), 1.60 (3H, d,  $J$  7.0,  $\text{CH}_3$ ), 0.98 (3H, d,  $J$  7.0,  $\text{CH}_3$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 143.6, 143.3, 139.0 (C), 123.7, 122.4, 114.5, 47.8 (CH), 12.9, 6.1 ( $\text{CH}_3$ );  $m/z$  (EI) 226 ( $\text{M}^+$ ), 151 (M- $\text{SC}_3\text{H}_7$ ), 121 (M- $\text{C}_3\text{H}_7\text{-NO}$ ), Found ( $\text{M}^+$ ) 258.0210,  $\text{C}_9\text{H}_{10}\text{SN}_2\text{SO}_5$  requires 258.0310; HPLC (hexane:EtOH, 60:40):  $R_t$  19.6 and 24.3 min.

#### **1-*tert*-Butylsulfanyl-2,4-dinitrobenzene** **272**.<sup>197</sup>



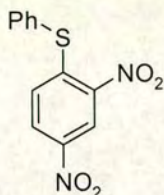
To a stirred solution of 2-methyl-propane-2-thiol sodium salt (1.17 g, 10.4 mmol) in ethanol (30 mL) was added a solution of *ortho*-chloro-2,4-dinitrobenzene **269** (4.00 g, 19.8 mmol) in ethanol (20 mL) and the reaction stirred at 40 °C for 5 h. After complete substrate consumption (TLC), the reaction mixture was poured into dilute HCl (2 %, 300 mL) and extracted with ether (3 x 200 mL). The combined organic extracts were dried ( $\text{MgSO}_4$ ), filtered and concentrated under reduced pressure. The crude product was purified by flash-column chromatography (ethyl acetate:hexane, 1:4) to afford *1-tert-butylsulfanyl-2-nitrobenzene* **272** as a yellow solid (1.75 g, 34.5 %).

$R_f$  (EtOAc:hexane, 4:1) 0.12; Mp 103.2-105.1 °C, Lit.<sup>197</sup> 104.0-107.0 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3106 (CH), 1604 (Ph), 1546, 1353 ( $\text{NO}_2$ );  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz)



8.91 (1H, d,  $J$  2.6, PhH), 8.39 (1H, dd,  $J$  8.8 and 2.6, PhH), 7.80 (1H, d,  $J$  8.8, PhH), 1.47 (9H, s, CH<sub>3</sub>);  $\delta_c$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 154.1, 146.2 (C), 134.8 (CH), 132.9 (C), 129.9, 123.2 (CH), 43.2 (C), 31.0 (CH<sub>3</sub>);  $m/z$  (EI) 256 (M<sup>+</sup>), 167 (M-SC(CH<sub>3</sub>)<sub>3</sub>), 121 (M-SC(CH<sub>3</sub>)<sub>3</sub>-NO<sub>2</sub>); HPLC (hexane:EtOH, 60:40): R<sub>t</sub> 18.4 min.

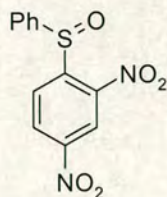
**1-(Phenylsulfanyl)-2,4-dinitrobenzene 273.**<sup>198</sup>



To a stirred solution of benzenethiol (8.51 g, 7.6 mL), NaHCO<sub>3</sub> (6.21 g, 73.9 mmol) in water (22 mL) was added a solution of and *ortho*-chloro-2,4-dinitrobenzene **269** (15.00 g, 73.9 mmol) in hot ethanol (92 mL) and the reaction heated at 65 °C. After 4 h, the solution was poured into water (175 mL) and stirred for 30 min at room temperature. The product was obtained by gravity filtration, washed several times with water and dried *in vacuo*. Recrystallisation (ethanol) afforded 1-(phenylsulfanyl)-2,4-dinitrobenzene **273** as yellow needle-like crystals (6.99 g, 34.3 %).

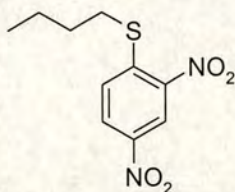
R<sub>f</sub> (EtOAc:Petrol, 3:2) 0.58; Bp 121.3-122.9 °C, Lit.<sup>199</sup> 122.0-123.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3101 (CH), 1591 (Ph), 1517, 1341(NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.07 (1H, d,  $J$  2.5, PhH), 8.11 (1H, dd,  $J$  9.1 and 2.5, PhH), 7.57 (5H, m, PhH), 6.98 (1H, d,  $J$  9.1, PhH);  $\delta_c$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 148.3, 14.2, 143.7 (C), 135.8, 131.0, 130.6 (CH), 128.9 (C), 128.7, 126.8, 121.3, 107.6 (CH);  $m/z$  (EI) 276 (M<sup>+</sup>), 199 (M-C<sub>6</sub>H<sub>5</sub>), 167 (M-SC<sub>6</sub>H<sub>5</sub>); HPLC (hexane:EtOH, 70:30): R<sub>t</sub> 20.1 min.



**1-Benzenesulfinyl-2,4-dinitrobenzene 277.**

To a stirred solution of sulfide **273** (990.0 mg, 3.6 mmol) in chloroform (20 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (690 mg, 4.0 mmol) in chloroform (12 mL). The reaction was stirred for 15 min then left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq. NaHCO<sub>3</sub> (50 mL) solution. The organic layer was separated, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *1-benzenesulfinyl-2,4-dinitrobenzene 277* as yellow crystals (570.0 mg, 53.9 %).

R<sub>f</sub> (EtOAc:Petrol, 3:2) 0.44; Mp 105.3-106.7 °C, Lit.<sup>200</sup> 106.5-107.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3984 (CH), 1585 (Ph), 1537, 1349 (NO<sub>2</sub>), 1066 (S=O);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.02 (1H, d, *J* 1.6, PhH), 8.80 (1H, dd, *J* 8.5 and 1.6, PhH), 8.01 (5H, m, Ph'H), 7.45 (1H, d, *J* 8.5, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 150.9, 149.3, 144.7, 143.7 (C), 132.2, 129.5, 129.1, 128.0, 126.7, 120.6 (CH); *m/z* (EI) 382 (M<sup>+</sup>), 215 (M-C<sub>6</sub>H<sub>5</sub>), 183 (M-SC<sub>6</sub>H<sub>5</sub>); HPLC (hexane:EtOH, 60:40): R<sub>t</sub> 44.6 and 101.6 min.

**1-Butylsulfanyl-2,4-dinitrobenzene 274.**<sup>201, 202</sup>

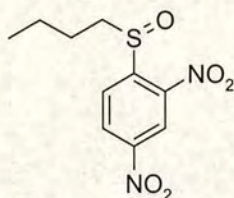
Under an inert atmosphere, butanethiol (6.67 g, 7.9 mL) was added to ethanol (220 mL) and stirred for 5 min after which time a solution of NaOH (2.90 g, 72.5 mmol) in water (22 mL) was added. This solution was added to a stirred solution of *ortho*-chloro-2,4-dinitrobenzene **269** (15.00 g, 73.9 mmol) in ethanol (95 mL) and the reaction heated under reflux for 10 min and filtered quickly. The



crystallised sulfide was collected by filtration and dried *in vacuo*, recrystallisation (ethanol) afforded *1-butylsulfinyl-2,4-dinitrobenzene* **274** as yellow needle-like crystals (6.05 g, 32.0 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.43; Mp 70.1-71.3 °C, Lit.<sup>203</sup> 72.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3185 (CH), 1592 (Ph), 1541, 1342 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.03 (1H, d,  $J$  1.9, PhH), 8.43 (1H, dd,  $J$  8.4 and 1.9, PhH), 7.11 (1H, d,  $J$  8.4, PhH), 2.99 (2H, t, SCH<sub>2</sub>), 1.63 (4H, m, CH<sub>2</sub>CH<sub>2</sub>Me), 0.95 (3H, t, CH<sub>3</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 144.2, 142.9, 138.2 (C), 127.8, 126.8, 120.8 (CH), 28.2, 27.5, 22.4 (CH<sub>2</sub>), 14.2 (CH<sub>3</sub>);  $m/z$  (EI) 256 (M<sup>+</sup>), 199 (M-C<sub>4</sub>H<sub>9</sub>), 167 (M-SC<sub>4</sub>H<sub>9</sub>).

#### 1-(Butane-1-sulfinyl)-2,4-dinitrobenzene **278**.



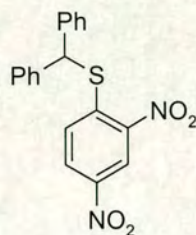
To a stirred solution of sulfide **274** (2.25 g, 8.8 mmol) in chloroform (40 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (1.59 g, 9.2 mmol) in chloroform (25 mL). The reaction was stirred for 15 min then left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq. NaHCO<sub>3</sub> (50 mL) solution. The organic layer was separated, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *1-(butane-1-sulfinyl)-2,4-dinitrobenzene* **278** as yellow crystals (1.62 g, 67.7 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.28; Mp 89.3-91.7 °C, (Found C, 43.89; H, 4.19; N, 10.48; C<sub>10</sub>H<sub>12</sub>N<sub>2</sub>O<sub>5</sub>S requires C, 44.11; H, 4.45; N, 10.29);  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3172 (CH), 1614 (Ph), 1555, 1347 (NO<sub>2</sub>), 1058 (S=O);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.18 (1H, d,  $J$  2.0, PhH), 8.75 (1H, dd,  $J$  8.3 and 2.0, PhH), 8.42 (1H, d,  $J$  8.3, PhH), 3.01 (1H, t, SCH<sub>2</sub>), 2.98 (1H, t, SCH<sub>2</sub>), 1.78 (4H, m, CH<sub>2</sub>CH<sub>2</sub>Me), 1.20 (3H, t, CH<sub>3</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 148.2, 145.1, 142.5 (C), 130.2, 128.7, 121.8 (CH), 48.3, 32.1, 23.5 (CH<sub>2</sub>), 15.0 (CH<sub>3</sub>);  $m/z$  (EI) 272 (M<sup>+</sup>), 215 (M-C<sub>4</sub>H<sub>9</sub>), 183 (M-SC<sub>4</sub>H<sub>9</sub>), 153 (M-SC<sub>4</sub>H<sub>9</sub>-NO),



Found ( $M^+$ ) 272.0458,  $C_{10}H_{12}N_2O_5S$  requires 272.0467; HPLC (hexane:EtOH, 75:25):  $R_t$  28.2 and 35.7 min.

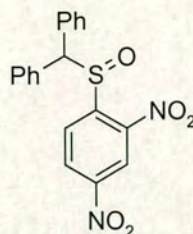
**1-Benzhydrylsulfanyl-2,4-dinitrobenzene 280.**<sup>193</sup>



To a pre-cooled solution of diphenylmethane **258** (4.44 g, 4.4 mL) and 2,4-Dinitrobenzenesulfenyl chloride **279** (6.17 g, 26.3 mmol) in anhydrous toluene (27 mL) under nitrogen was added drop-wise a solution of triethylamine (2.69 g, 3.7 mL) in anhydrous toluene (6 mL) and the reaction stirred for 6 h. The mixture was filtered and the filtrate concentrated under reduced pressure to yield an orange oil, which crystallised when treated with petrol. The crystals were collected under gravity filtration and recrystallised (ethanol) to afford *1-benzhydrylsulfanyl-2,4-dinitrobenzene 280* as pale orange crystals (4.03 g, 41.9 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.42; Mp 142.5 °C, Lit.<sup>204</sup> 141.0-143.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3097 (CH), 1584 (Ph), 1528, 1338 ( $\text{NO}_2$ );  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 8.91 (1H, d,  $J$  1.9, PhH), 8.87 (1H, dd,  $J$  8.8 and 1.9, PhH), 8.03 (1H, d,  $J$  8.8, PhH), 7.76 (8H, m, Ph'H), 7.51 (2H, m, Ph'H), 4.84 (1H, s, CH);  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 148.1, 145.2, 144.3, 140.9 (C), 133.6, 131.5, 128.9, 123.1, 52.2 (CH);  $m/z$  (EI) 366 ( $M^+$ ), 167 ( $M-\text{SCH}(\text{C}_6\text{H}_5)_2$ ), 121 ( $M-\text{SCH}(\text{C}_6\text{H}_5)_2-\text{NO}_2$ ).

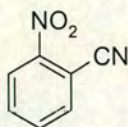


**1-(Diphenyl-methanesulfinyl)-2,4-dinitrobenzene 281.**

To a stirred solution of sulfide **280** (1.32 g, 3.6 mmol) in chloroform (20 mL) at -10 °C was added a solution of *meta*-chloroperbenzoic acid (690.0 mg, 4.0 mmol) in chloroform (12 mL). The reaction was stirred for 15 min then left to stand overnight at room temperature, whereafter it was filtered and washed twice with a sat. aq. NaHCO<sub>3</sub> (50 mL) solution. The organic layer was separated, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *1-diphenyl-methanesulfinyl-2,4-dinitrobenzene 281* as yellow crystals (0.57 g, 41.4 %).

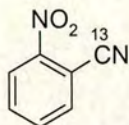
R<sub>f</sub> (EtOAc:Petrol, 1:1) 0.34; Mp 110.2-111.8 °C, Lit.<sup>205</sup> 113.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3102 (CH), 1595 (Ph), 1541, 1341 (NO<sub>2</sub>), 1052 (S=O);  $\delta_{\text{H}}((\text{CD}_3)_2\text{SO}, 200 \text{ MHz})$  9.41 (1H, d, *J* 2.1, PhH), 9.23 (1H, dd, *J* 9.1 and 2.1, PhH), 8.76 (1H, d, *J* 9.1, PhH), 7.85 (8H, m, Ph'H), 7.22 (2H, m, Ph'H), 5.05 (1H, s, CH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{SO}, 63 \text{ MHz})$  152.1, 150.6, 147.2 (C), 134.8, 130.1, 128.5, 128.0, 122.2, 120.1, 62.2 (CH); *m/z* (EI) 382 (M<sup>+</sup>), 183 (M-SCH(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub>), 137 (M-SCH(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub>-NO<sub>2</sub>); HPLC (hexane:EtOH, 50:50): R<sub>t</sub> 22.7 and 29.1 min.



6.6 Synthesis of  $^{13}\text{C}$ -2-nitro-benzonitrile.<sup>206, 207</sup>2-Nitro-benzonitrile **281**.

Under a stream of nitrogen, copper (I) cyanide (172.8 mg, 1.9 mmol) was added to DMF (2 mL) and the solution stirred for 10 min, after which time *ortho*-chloro-nitrobenzene **341** (252.8 mg, 1.6 mmol) was added. After 1 h, the reaction was heated to 45 °C for *ca.* 3 h then left stirring overnight at room temperature. The dark mixture was diluted with ethyl acetate (15 mL) and filtered through a Celite<sup>TM</sup> pad. The filtrate was washed with water (30 mL) and brine (30 mL), dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 2:3) afforded 2-nitro-benzonitrile **281** as a pale yellow solid (99.1 mg, 41.6 %).

$R_f$  (EtOAc:Petrol, 2:3) 0.33; Mp 110.1-110.9 °C, Lit.<sup>208</sup> 111.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3081 (CH), 2252 (CN), 1625 (Ph), 1540, 1360 (NO<sub>2</sub>);  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 200 MHz) 8.84 (1H, dd, *J* 7.7 and 1.5, PhH), 7.95 (3H, m, PhH);  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 63 MHz) 147.9, 135.2, 133.6, 133.1, 124.9 (CH), 113.8, 107.5 (C); *m/z* (EI) 148 (M<sup>+</sup>), 118 (M-NO), 102 (M-NO<sub>2</sub>), 75 (M-NO<sub>2</sub>-HCN); GC:  $R_t$  12.0 min, oven temp 120°C.

 $^{13}\text{C}$ -2-Nitro-benzonitrile **342**.

The procedure outlined above with  $^{13}\text{C}$ -labelled copper (I) cyanide (247.2 mg, 2.7 mmol) in DMF (2 mL) and *ortho*-chloro-nitrobenzene **341** (2.3 mmol) was followed. Purification by flash-column chromatography (ethyl acetate:petrol, 2:3) afforded  $^{13}\text{C}$ -2-nitro-benzonitrile **342** as a pale yellow solid (188.7 mg, 55.1 %).

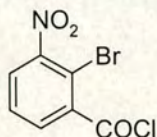


$R_f$  (EtOAc:Petrol, 2:3) 0.33;  $\delta_C$  (CDCl<sub>3</sub>, 63 MHz) 113.6 (C), enhanced signal;  $m/z$  (EI) 149 (M<sup>+</sup>), 117 (M-NO), 101 (M-NO<sub>2</sub>), 74 (M-NO<sub>2</sub>-HCN); GC:  $R_t$  12.0 min, oven temp 120°C; Data as in **281**.

## 6.7 Synthesis of 2-<sup>13</sup>C-3-nitro-phthalonitrile.

### 6.7.1 Method I.

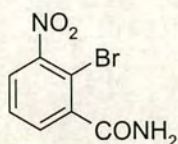
#### 2-Bromo-3-nitro-benzoyl chloride **349a**.



Under an inert atmosphere, 2-bromo-3-nitro-benzoic acid **348a** (1.00 g, 4.1 mmol, pre-dried at 100 °C for 1 h) was heated under reflux with thionyl chloride (17.44 g, 10.7 mL) for *ca.* 24 h. Excess thionyl chloride was removed *in vacuo* to yield an oil which afforded on cooling pale yellow needle-like crystals of 2-bromo-3-nitro-benzoyl chloride **349a** as a pale yellow solid (895.8 mg, 82.6 %).

Mp 66.1-66.5 °C, Lit.<sup>209</sup> 66.0-66.5 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3076 (CH), 1770 (C=O), 1585 (Ph), 1534, 1355 (NO<sub>2</sub>);  $\delta_H$  (CDCl<sub>3</sub>, 200 MHz) 8.11 (1H, dd, *J* 8.8 and 1.9, PhH), 8.07 (1H, dd, *J* 8.2 and 1.9, PhH), 7.94 (1H, dd, *J* 8.8 and 8.2, PhH);  $\delta_C$  (CDCl<sub>3</sub>, 63 MHz) 167.1, 151.3 (C), 142.2 (CH), 139.8 (C), 135.1, 130.9 (CH), 121.2 (C);  $m/z$  (EI) 264 (M<sup>+</sup>), 184 (M-Br), 138 (M-Br-NO<sub>2</sub>).

#### 2-Bromo-3-nitro-benzamide **350a**.



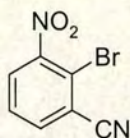
2-Bromo-3-nitro-benzoyl chloride **349a** (850.0 mg, 3.2 mmol) was introduced slowly into a cooled (0 °C) beaker of concentrated ammonia solution (5 mL) and the



resulting heterogeneous mixture stirred at 25 °C for 24 h. The diamide was collected by gravity filtration, dried *in vacuo* and the resulting solid purified by recrystallisation (water) to afford **2-bromo-3-nitro-benzamide 350a** as white crystals (573.1 mg, 73.1 %).

Mp 170.2-171.3 °C, Lit.<sup>210</sup> 170.0-171.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3384, 3187 (NH<sub>2</sub>), 3071 (CH), 1657 (C=O, NH), 1533, 1374 (NO<sub>2</sub>);  $\delta_{\text{H}}(\text{CDCl}_3, 200 \text{ MHz})$  8.34 (1H, dd, *J* 8.3 and 2.3, PhH), 8.13 (1H, br s, CONH<sub>2</sub>), 8.01 (1H, dd, *J* 7.9 and 2.3, PhH), 7.83 (1H, br s, CONH<sub>2</sub>), 7.67 (1H, dd, *J* 8.3 and 7.9, PhH);  $\delta_{\text{C}}(\text{CDCl}_3, 63 \text{ MHz})$  168.6, 151.8, 138.9 (C), 131.0, 130.0, 125.5 (CH), 112.6 (C); *m/z* (ES<sup>+</sup>) 246 (MH<sup>+</sup>), 165 (M-Br), 135 (M-Br-NO<sub>2</sub>).

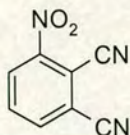
### 2-Bromo-3-nitro-benzonitrile 351a.



2-Bromo-3-nitro-benzamide **350a** (550.0 mg, 2.2 mmol, pre-dried at 110 °C for 2 h) was heated under reflux with excess phosphorus oxychloride (*ca.* 5 mL) for 4 h. The resulting yellow solution was concentrated under reduced pressure to afford a light yellow solid. Recrystallisation (water) afforded **2-bromo-3-nitro-benzonitrile 351a** as pale yellow crystals (357.6 mg, 71.6 %).

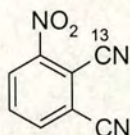
Mp 135.4-136.8 °C, Lit.<sup>211</sup> 136.0-137.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3072 (CH), 2235 (CN), 1590 (Ph), 1539, 1358 (NO<sub>2</sub>);  $\delta_{\text{H}}(\text{CDCl}_3, 200 \text{ MHz})$  8.32 (1H, dd, *J* 8.1 and 1.5, PhH), 8.03 (1H, dd, *J* 7.8 and 1.5, PhH), 7.82 (1H, dd, *J* 8.1 and 7.8, PhH);  $\delta_{\text{C}}(\text{CDCl}_3, 63 \text{ MHz})$  150.7 (C), 138.3, 130.2, 129.7 (CH), 122.5, 117.3, 116.4 (C); *m/z* (EI) 227 (M<sup>+</sup>), 147 (M-Br), 101 (M-Br-NO<sub>2</sub>), 74 (M-Br-NO<sub>2</sub>-HCN).



**3-Nitro-phthalonitrile 238.**

Under a stream of nitrogen, copper (I) cyanide (252.0 mg, 2.8 mmol) was added to dimethylacetamide (2 mL) and the solution stirred for 10 min, after which time 2-bromo-3-nitro-benzonitrile **351a** (522.1 mg, 2.3 mmol) was added. After 1 h, the reaction was heated to 45 °C for *ca.* 3 h then left stirring overnight at room temperature. The dark mixture was diluted with ethyl acetate (15 mL) and filtered through a Celite<sup>TM</sup> pad. The filtrate was washed with water (30 mL) and brine (30 mL), dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 3-nitro-phthalonitrile **238** as a pale yellow solid (188.7 mg, 55.1 %).

R<sub>f</sub> (EtOAc:Petrol, 1:1) 0.52; Mp 160.2-161.8 °C; *m/z* (EI) 173 (M<sup>+</sup>), 143 (M-NO), 127 (M-NO<sub>2</sub>), 73 (M-NO<sub>2</sub>-2HCN); Data as in **238**.

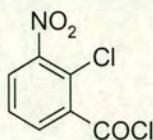
**2-<sup>13</sup>C-3-Nitro-phthalonitrile 343.**

The procedure outlined above with <sup>13</sup>C-labelled copper (I) cyanide (248.1 mg, 2.7 mmol) in dimethylacetamide (2mL) and 2-bromo-3-nitro-benzonitrile **351a** (522.1 mg, 2.3 mmol) was followed. Purification by flash-column chromatography, (ethyl acetate:petrol, 1:1) afforded 2-<sup>13</sup>C-3-nitro-phthalonitrile **343** as a pale yellow solid (213.4 mg, 62.3 %).

R<sub>f</sub> (EtOAc:Petrol, 1:1) 0.52; Mp 159.4-161.8 °C; δ<sub>C</sub> ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 113.4 (C), enhanced signal; *m/z* (EI) 174 (M<sup>+</sup>), 144 (M-NO), 128 (M-NO<sub>2</sub>); Data as in **238**.

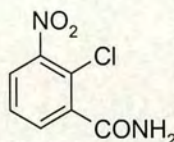


## 6.7.2 Method II.

**2-Chloro-3-nitro-benzoyl chloride 349b.**

Under an inert atmosphere, 2-chloro-3-nitro-benzoic acid **348b** (1.00 g, 5.0 mmol, pre-dried at 100 °C for 1 h) was heated under reflux with thionyl chloride (17.44 g, 10.7 mL) for *ca.* 24 h. Excess thionyl chloride was removed *in vacuo* to yield an oil which afforded on cooling 2-chloro-3-nitro-benzoyl chloride **349b** as a pale yellow solid (906.4 mg, 82.4 %).

Mp 56.8-57.2 °C, Lit.<sup>212</sup> 57.0-58.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3082 (CH), 1763 (C=O), 1590 (Ph), 1538, 1355 (NO<sub>2</sub>);  $\delta_{\text{H}}(\text{CDCl}_3, 200 \text{ MHz})$  8.34 (1H, dd, *J* 7.5 and 1.6, PhH), 8.22 (1H, dd, *J* 7.9 and 1.6, PhH), 8.01 (1H, dd, *J* 7.9 and 7.5, PhH);  $\delta_{\text{C}}(\text{CDCl}_3, 63 \text{ MHz})$  168.5, 149.7 (C), 140.7 (CH), 132.0, 128.6 (C), 127.1, 126.9 (CH); *m/z* (EI) 220 (M<sup>+</sup>), 174 (M-NO<sub>2</sub>), 139 (M-NO<sub>2</sub>-Cl).

**2-Chloro-3-nitro-benzamide 350b.**

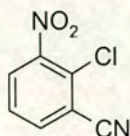
2-Chloro-3-nitro-benzoyl chloride **349b** (880.0 mg, 4.0 mmol) was introduced slowly into a cooled (0 °C) beaker of concentrated ammonia solution (5 mL) and the resulting heterogeneous mixture stirred at 25 °C for 24 h. The diamide was collected by gravity filtration, dried *in vacuo* and the resulting solid purified by recrystallisation (water) to afford 2-chloro-3-nitro-benzamide **350b** as pale yellow crystals (582.1 mg, 72.4 %).

Mp 146.0-148.2 °C, Lit.<sup>213</sup> 147.0-148.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3367, 3186 (NH<sub>2</sub>), 1656 (C=O, NH), 1595 (Ph), 1530, 1355 (NO<sub>2</sub>);  $\delta_{\text{H}}(\text{CDCl}_3, 200 \text{ MHz})$  8.29 (1H, dd, *J* 7.7



and 1.8, PhH), 8.25 (1H, br s, CONH<sub>2</sub>), 8.17 (1H, br s, CONH<sub>2</sub>), 8.19 (1H, dd, *J* 7.7 and 1.8, PhH), 8.00 (1H, dd, *J* 7.9 and 7.7, PhH);  $\delta_c$  (CDCl<sub>3</sub>, 63 MHz) 169.5, 150.9, 135.2 (C), 134.5, 128.6 (CH), 127.7 (C), 126.5 (CH); *m/z* (ES<sup>+</sup>) 221 (MH<sup>+</sup>), 174 (M-NO<sub>2</sub>), 139 (M-NO<sub>2</sub>-Cl).

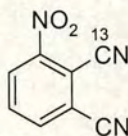
### 2-Chloro-3-nitro-benzonitrile **351b**.



2-Chloro-3-nitro-benzamide **350b** (550.0 mg, 2.7 mmol, pre-dried at 110 °C for 2 h) was heated under reflux with excess phosphorus oxychloride (*ca.* 5 mL) for 4 h. The resulting yellow solution was concentrated under reduced pressure to yield a light yellow solid. Recrystallisation (water) afforded 2-chloro-3-nitro-benzonitrile **351b** as pale yellow crystals (396.3 mg, 80.2 %).

Mp 101.4-102.8 °C, Lit.<sup>214</sup> 97.0-101.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3094 (CH), 2246 (CN), 1602 (Ph), 1535, 1360 (NO<sub>2</sub>);  $\delta_H$  (CDCl<sub>3</sub>, 200 MHz) 8.40 (1H, dd, *J* 8.2 and 1.5, PhH), 8.31 (1H, dd, *J* 7.8 and 1.5, PhH), 7.80 (1H, dd, *J* 8.2 and 7.8, PhH);  $\delta_c$  (CDCl<sub>3</sub>, 63 MHz) 148.2 (C), 138.4 (CH), 135.7 (C), 128.4, 126.2 (CH), 115.2, 114.9 (C); *m/z* (EI) 183 (M<sup>+</sup>), 137 (M-NO<sub>2</sub>), 102 (M-NO<sub>2</sub>-Cl).

### 2-<sup>13</sup>C-3-Nitro-phthalonitrile **343**.



Under a stream of nitrogen, <sup>13</sup>C-labelled copper (I) cyanide (247.5 mg, 2.7 mmol) was added to dimethylacetamide (2 mL) and the solution stirred for 10 min, after which time 2-chloro-3-nitro-benzonitrile **351b** (549.2 mg, 3.0 mmol) was added. After 1 h, the reaction was heated to 45 °C for *ca.* 3 h then left stirring overnight at room temperature. The dark mixture was diluted with ethyl acetate (15 mL) and filtered through a Celite<sup>TM</sup> pad. The filtrate was washed with water (30 mL) and brine (30 mL), dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure.

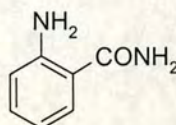


Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 2-<sup>13</sup>C-3-nitro-phthalonitrile **343** as a pale yellow solid (354.0 mg, 68.2 %).

R<sub>f</sub> (EtOAc:Petrol, 1:1) 0.50; Mp 159.9-160.8 °C; δ<sub>C</sub> ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 113.4 (C), enhanced signal; m/z (EI) 174 (M<sup>+</sup>), 144 (M-NO), 128 (M-NO<sub>2</sub>).

## 6.8 Intermediate synthesis.

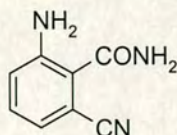
### 2-Amino-benzamide **284**.



Zinc powder (1.50 g, 23.1 mmol) was added to a stirred suspension of 2-nitro-benzonitrile **282** (1.00 g, 6.8 mmol) and ammonium chloride (75.0 mg, 14.0 mmol) in 20 % ethanol (20 mL) over a period of 20 min and the resulting mixture stirred for a further 20 min. The precipitated solid was separated by filtration and washed with ethanol. The deep orange/yellow filtrate obtained was diluted with water and extracted with ethyl acetate:ether (1:1, 2 x 40 mL). The combined organic extracts were dried (MgSO<sub>4</sub>), filtered and reduced *in vacuo*. Purification by flash-column chromatography (activated alumina: ethyl acetate:chloroform, 3:2) afforded a yellow coloured band eluting first followed by a orange/red coloured band, which was collected. The red coloured solution was reduced *in vacuo* and the resulting solid recrystallised twice (toluene) to afford 2-amino-benzamide **284** as white crystals (669.6 mg, 72.4 %).

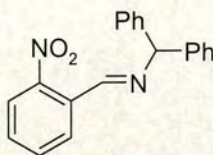
R<sub>f</sub> (EtOAc:CHCl<sub>3</sub>, 3:2) 0.23; Mp 111.8-112.8 °C, Lit.<sup>215</sup> 113.0-114.0 °C; ν<sub>max</sub>(KBr)/cm<sup>-1</sup> 3411, 3324 (NH<sub>2</sub>), 3201 (CH), 1659 (C=O), 1628 (NH), 1609 (Ph); δ<sub>H</sub> (CDCl<sub>3</sub>, 200 MHz) 7.36 (1H, dd, *J* 8.1 and 1.5, PhH), 7.34 (1H, td, *J* 8.3, 7.8 and 1.5, PhH), 7.20 (2H, m, PhH), 5.91 (2H, br s, CONH<sub>2</sub>), 4.02 (2H, br s, NH<sub>2</sub>); δ<sub>C</sub> (CDCl<sub>3</sub>, 63 MHz) 171.6, 149.3 (C), 132.9, 127.9, 117.3, 116.3 (CH), 113.9 (C); m/z (EI) 136 (M<sup>+</sup>), 92 (M-CONH<sub>2</sub>); GC: R<sub>t</sub> 15.0 min, oven temp 125 °C.



**2-Amino-6-cyano-benzamide 289.**

Zinc powder (75.0 mg, 11.5 mmol) was added to a stirred suspension of 3-nitro-phthalonitrile **238** (500.0 mg, 3.4 mmol) and ammonium chloride (37.5 mg, 14.0 mmol) in 20 % ethanol (20 mL) over a period of 20 min and the resulting mixture stirred for a further 20 min. The precipitated solid was separated by filtration and washed with ethanol. The deep orange/yellow filtrate obtained was diluted with water and extracted with ethyl acetate:ether (1:1, 2 x 40 mL). The combined organic extracts were dried (MgSO<sub>4</sub>), filtered and reduced *in vacuo*. Purification by flash-column chromatography (activated alumina: ethyl acetate:*iso*-propanol, 1:1) afforded a solid which recrystallised twice (toluene) to afford *2-amino-6-cyano-benzamide 289* as yellow crystals (270.4 mg, 49.4 %).

$R_f$  (EtOAc:*iso*-propanol, 3:2) 0.21; Mp >350.0 °C; Lit.<sup>66</sup> 204.0 °C  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3297, 3145 (NH<sub>2</sub>), 2988 (CH), 2219 (CN), 1655 (C=O), 1633 (NH), 1601 (Ph);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 360 MHz) 9.92 (1H, br s, CONH), 9.47 (1H, br s, CONH), 7.32 (1H, dd,  $J$  8.3 and 7.3, PhH), 7.12 (1H, dd,  $J$  7.3 and 0.8, PhH), 6.80 (1H, dd,  $J$  8.3 and 0.8, PhH), 6.13 (2H, br s, NH<sub>2</sub>);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 90 MHz) 164.7, 145.9, 134.5 (C), 133.6, 118.5, 111.80 (CH), 111.7 (C), 109.2 (CH);  $m/z$  (EI) 161 (M<sup>+</sup>), 144 (M-NH<sub>3</sub>), 117 (M-CONH<sub>2</sub>); GC:  $R_t$  21.8 min, oven temp 150 °C.

**Benzhydryl-(1-(2-nitro-phenyl)-methylene)-amine 358.**<sup>153</sup>

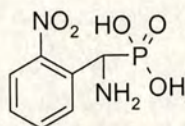
To a stirred solution of diphenylmethylaniline **357** (3.43 g, 3.4 mL) and anhydrous KCO<sub>3</sub> in toluene (50 mL) was added a solution of *ortho*-nitro-benzaldehyde **356** (3.00g, 19.8 mmol) in toluene (50 mL). The mixture was stirred at room temperature



for 1 h, filtered and concentrated under reduced pressure to afford a viscous oil which crystallised upon standing to afford *benzhydryl-(1-(2-nitro-phenyl)-methylene)-amine 358* as a yellow solid (6.16 g, 98.3 %).

Mp 96.2-97.6 °C, (Found C, 75.79; H, 4.8; N, 8.58; C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub> requires C, 75.92; H, 5.1; N, 8.41);  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  1640 (C=N), 1631 (Ph), 1545, 1365 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.89 (1H, s, CH=N), 8.22 (1H, dd, *J* 7.3 and 1.6, PhH), 8.01 (1H, dd, *J* 8.0 and 1.3, PhH), 7.67 (1H, td, *J* 7.4, 7.2 and 1.4, PhH), 7.55 (1H, td, *J* 8.1, 7.4 and 1.6, PhH), 7.33 (10H, m, PhH), 5.73 (1H, s, NCH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 156.7 (CH), 148.8, 142.9 (C), 133.3 (CH), 131.0 (C), 130.7, 130.0, 128.4, 127.5, 127.1, 124.2, 77.4 (CH); *m/z* (EI) 316 (M<sup>+</sup>), 239 (M-C<sub>6</sub>H<sub>5</sub>), Found (M<sup>+</sup>) 316.0911, C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub> requires 316.1218.

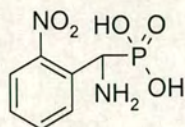
### 1-Amino-2-nitro-benzylphosphonic acid **359**.<sup>154</sup>



A stirred solution of *benzhydryl-(1-(2-nitro-phenyl)-methylene)-amine 358* (6.16 g, 19.5 mmol) and diethyl phosphite (2.97 g, 21.5 mmol) in toluene (50 mL) was heated to 130-140 °C. After 30 min, excess concentrated hydrochloric acid was added and the solution heated under reflux for 3 h. Upon cooling, a solid crystallised out of solution which was collected by filtration and dried *in vacuo* to afford *1-amino-2-nitro-benzylphosphonic acid 359* as white crystals (3.80 g, 84.0 %).

Mp 243.9-246.8 °C, Lit.<sup>154</sup> 245.0-247.0;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3208 (br, OH), 1645 (Ph), 1559, 1372 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.34 (2H, br s, POOH), 7.76 (1H, dd, *J* 8.4 and 1.6, PhH), 7.37 (3H, m, PhH), 5.61 (1H, s, CHP);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 138.6 (C), 128.8 (CH), 128.3 (C), 127.5, 57.2 (CH); *m/z* (EI) 232 (M<sup>+</sup>), 216 (M-NH<sub>2</sub>).



Attempted Cleavage of phosphonic acid **359**.<sup>154</sup>

1-Amino-2-nitro-benzylphosphonic acid **359** (3.70, 15.9 mmol) was dissolved in aqueous sodium hydroxide (5.10 g in 266 mL of H<sub>2</sub>O) and heated under reflux for 2 h. Upon cooling, the solution was neutralised with aq. HCl (6M) and the precipitate removed by filtration and dried *in vacuo* to afford the *starting material* **359** as white crystals (3.55 g, 95.9 %).

Mp 244.0-246.8 °C; (EI) 232 (M<sup>+</sup>); Data as in **359**.



## 7. Experimental : Biotransformations.

### 7.1 General Experimental.

Instrumentation, GCMS, HPLC and LCMS conditions are shown in Section 6.1.

*Saccharomyces cerevisiae* (Sigma Type II, YSC-2) described in this work was purchased from Sigma and was washed with pre-cooled acetone (-18 °C) prior to use.

The *Rhodococcus* species (freeze-dried whole cells) was given to the group by Zylepsis and used as received. The phosphate buffer used in these biotransformations was made up from the following: Na<sub>2</sub>HPO<sub>4</sub> (6 g/L), KH<sub>2</sub>PO<sub>4</sub> (3 g/L) and NaCl (0.5 g/L) in distilled water, pH *ca.* 7.0.

NAD(P)H dehydrogenase (Old Yellow Enzyme II, NRase II), Thioredoxin reductase (NRase III) and Lipoamide dehydrogenase (NRase I) were isolated and purified from *S. cerevisiae* within our research laboratories by Marina Alexeeva.

Glutathione reductase was purchased from Sigma as a suspension in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution, pH 7.0.

L-Lactate dehydrogenase from *S. cerevisiae* and P-450 Cytochrome reductase from *Pascillus bacterium* were gifts from Prof. Chapman in the Chemistry Department.

#### Orbital Shaker.

A 625 Model incubator shaker was used for all incubations, which was set at a temperature of 30 °C.



## 7.2 Preparation of acetone-washed *S. cerevisiae*.<sup>69</sup>

*S. cerevisiae* (125.00 g) was added to pre-cooled acetone (-18 °C) and the resulting suspension was stirred gently for 15 min. Once the *S. cerevisiae* had settled out, the acetone was decanted and the procedure was repeated. The cell mass was collected by filtration and dried under vacuum.

## 7.3 General Procedure I : *S. cerevisiae* catalysed reduction of nitroarenes.<sup>66, 69</sup>

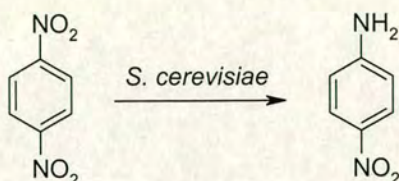
The 'acetone-washed' *S. cerevisiae* (25.00 g) was suspended in distilled water (100 mL) and incubated in an orbital shaker at 30 °C and 200 rpm for *ca.* 1 h. The substrate (250.0 mg) was dissolved in a minimum amount of DMSO or dioxane and added to the *S. cerevisiae* suspension. The reduction of the substrate was monitored by GCMS of 1 mL samples withdrawn at regular time intervals from the reaction mixture. Each sample was added to ethyl acetate (1 mL) in an eppendorf tube and the resulting biphasic solution was mixed using a rotameter and then microcentrifuged to separate the layers. The organic layer (800 µL) containing the product(s) was removed and further diluted with ethyl acetate (500 µL) and subjected to GCMS analysis.

Upon complete substrate consumption, the *S. cerevisiae* suspension was continuously extracted with chloroform overnight. The chloroform extract was dried (MgSO<sub>4</sub>), filtered and concentrated *in vacuo* to yield the crude product. Further purification of the crude was achieved by flash-column chromatography or preparative HPLC.

Quantities of substrate used, as well as reaction times, purification methods and yields are given for each individual biotransformation.

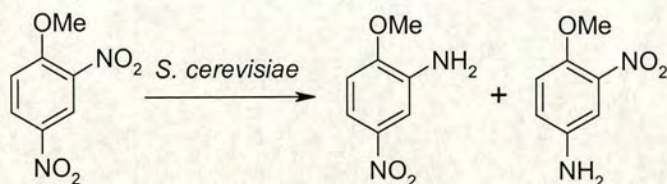


## 7.3.1 Reduction of nitroarenes.

4-Nitro-aniline **321**.

According to General Procedure I, 1,4-dinitrobenzene **320** (250.0 mg, 1.5 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 6.3 h. After work-up, purification by flash-column chromatography (dichloromethane:petrol, 7:3) afforded 4-nitro-aniline **321** as an orange solid (91.0 mg, 44.8 %).

$R_f$  (DCM:Petrol, 7:3) 0.15; Mp 147.9-148.5 °C, Lit.<sup>216</sup> 147.5-150.5 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3392 (NH<sub>2</sub>), 1600 (Ph), 1522, 1348 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.93 (2H, d,  $J$  7.0, PhH), 6.70 (2H, br s, NH<sub>2</sub>), 6.58 (2H, d,  $J$  7.0, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 155.8, 135.7 (C), 126.5, 112.5 (CH);  $m/z$  (EI) 138 (M<sup>+</sup>), 122 (M-NH<sub>2</sub>), 92 (M-NH<sub>2</sub>-NO); GC: Rt 26.4 min, oven temp 150 °C.

2-Amino-4-nitro-anisole **331** and 4-Amino-2-nitro-anisole **332**.

According to General Procedure I, 2,4-dinitro-anisole **330** (250.0 mg, 3.0 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 7 d. After work-up, purification by flash-column chromatography (dichloromethane:petrol, 7:3) afforded 2-amino-4-nitro-anisole **331** as an orange/yellow solid (105.0 mg, 49.8 %) and 4-amino-2-nitro-anisole **332** as an orange gum (20.0 mg, 10.0 %).

2-Amino-4-nitro-anisole:  $R_f$  (DCM:Petrol, 7:3) 0.35; Mp 116.8 °C, Lit.<sup>66</sup> 118.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3467, 3370 (NH<sub>2</sub>), 2923 (CH), 1624 (Ph), 1513, 1339 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.64 (1H, dd,  $J$  8.9 and 2.7, PhH), 7.53 (1H, d,  $J$  2.7, PhH),

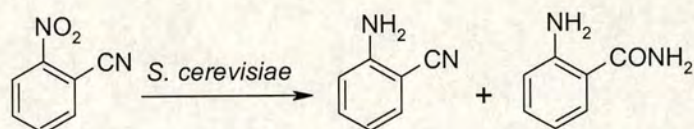


6.79 (1H, d,  $J$  8.9, PhH), 4.0 (1H, br s, NH<sub>2</sub>), 3.93 (3H, s, OCH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 151.8, 141.7, 136.5 (C), 114.7, 108.8, 108.7 (CH), 55.9 (CH<sub>3</sub>);  $m/z$  (EI) 168 (M<sup>+</sup>), 122 (M-NO<sub>2</sub>), 122 (M-OCH<sub>3</sub>-NH<sub>3</sub>); GC: R<sub>t</sub> 6.7 min, oven temp 150 °C.

4-Amino-2-nitro-anisole: R<sub>f</sub> (DCM:Petrol, 7:3) 0.12;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 2995, 2907 (NH<sub>2</sub>), 1605 (Ph), 1529, 1374 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.17 (1H, d,  $J$  2.8, PhH), 6.91 (1H, d,  $J$  8.8, PhH), 6.86 (2H, dd,  $J$  8.8 and 2.8, PhH), 3.87 (3H, s, OCH<sub>3</sub>), 3.63 (1H, br s, NH<sub>2</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 145.9, 139.9 (C), 120.8, 115.5, 111.5 (CH), 70.5, 57.1 (C);  $m/z$  (EI) 168 (M<sup>+</sup>), 122 (M-NO<sub>2</sub>), 122 (M-OCH<sub>3</sub>-NH<sub>3</sub>); GC: R<sub>t</sub> 5.8 min, oven temp 150 °C.

### 7.3.2 Reduction of cyanonitroarene.

#### 2-Amino-benzonitrile **283** and 2-Amino-benzamide **284**.

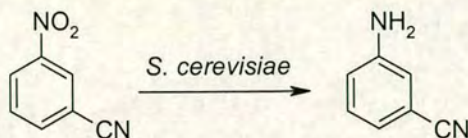


According to General Procedure I, 2-nitro-benzonitrile **282** (250.0 mg, 1.7 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 2 h. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 2-amino-benzonitrile **283** as a cream solid (23.0 mg, 10.5 %) and 2-amino-benzamide **284** as a yellow solid (82.0 mg, 35.0 %).

2-Amino-benzonitrile: R<sub>f</sub> (EtOAc:Petrol, 1:1) 0.40; Mp 46.8-47.9 °C, Lit.<sup>217</sup> 47.0-49.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3434, 3387 (NH<sub>2</sub>), 2379 (CN), 1600 (Ph);  $\delta_H$  (CDCl<sub>3</sub>, 200 MHz) 7.30 (2H, m, PhH), 6.78 (1H, td,  $J$  8.2, 8.0 and 1.4, PhH), 6.52 (1H, dd,  $J$  8.0 and 1.4, PhH), 5.6 (2H, br s, NH<sub>2</sub>);  $\delta_C$  (CDCl<sub>3</sub>, 63 MHz) 150.9 (C), 132.2, 131.5 (CH), 116.4 (C), 115.2, 113.9 (CH), 93.4 (C);  $m/z$  (EI) 118 (M<sup>+</sup>), 91 (M-HCN), 64 (M-2HCN); GC: R<sub>t</sub> 4.3 min, oven temp 125 °C.

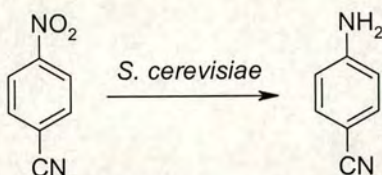
2-Amino-benzamide: R<sub>f</sub> (EtOAc:Petrol, 1:1) 0.27; Mp 112.4-113.9 °C;  $m/z$  (EI) 136 (M<sup>+</sup>), 92 (M-CONH<sub>2</sub>); GC: R<sub>t</sub> 15.1 min, oven temp 125 °C; Data as in **284**.



**3-Amino-benzonitrile 286.**

According to General Procedure I, 3-nitro-benzonitrile **285** (250.0 mg, 1.7 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 7 d. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 3-amino-benzonitrile **286** as a yellow solid (79.0 mg, 37.6 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.45; Mp 51.0-52.0 °C, Lit.<sup>218</sup> 52.0-54.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3422, 3360 (NH<sub>2</sub>), 3059 (CH), 2250 (CN), 1600 (Ph);  $\delta_H$  (CDCl<sub>3</sub>, 200 MHz) 7.22 (1H, td,  $J$  8.1, 7.9 and 1.5, PhH), 7.15 (1H, dd,  $J$  1.5, PhH), 6.87 (2H, m, PhH), 3.92 (2H, br s, NH<sub>2</sub>);  $\delta_C$  (CDCl<sub>3</sub>, 63 MHz) 146.8, 135.6 (C), 129.8, 121.7, 119.0, 117.2 (CH), 112.6 (C);  $m/z$  (ES<sup>+</sup>) 119 (MH<sup>+</sup>), 91 (M-HCN); GC:  $R_t$  10.3 min, oven temp 150 °C.

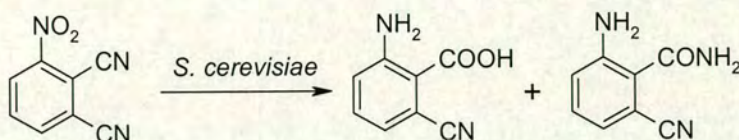
**4-Amino-benzonitrile 288.**

According to General Procedure I, 4-nitro-benzonitrile **287** (250.0 mg, 1.7 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 24 h. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 4-amino-benzonitrile **288** as a cream solid (126.0 mg, 61.4 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.46; Mp 86.3-88.0 °C, Lit.<sup>219</sup> 86.0-89.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3382, 3211 (NH<sub>2</sub>), 2919 (CH), 2219 (CN), 1605 (Ph), 832 (*para*-disubstituted Ph ring);  $\delta_H$  (CDCl<sub>3</sub>, 200 MHz) 7.38 (2H, d,  $J$  8.7, PhH), 6.63 (2H, d,  $J$  8.7, PhH), 4.15 (2H, br s, NH<sub>2</sub>);  $\delta_C$  (CDCl<sub>3</sub>, 63 MHz) 153.7 (C), 133.7 (CH), 120.1 (C), 114.3 (CH), 99.8 (C);  $m/z$  (CI<sup>+</sup>) 141 (MNa<sup>+</sup>); GC:  $R_t$  3.1 min, oven temp 150 °C.



## 7.3.3 Reduction of dicyanonitroarenes.

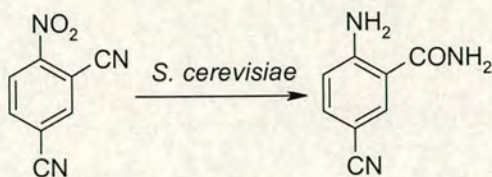
2-Amino-6-cyano-benzoic acid **290** and 2-Amino-6-cyano-benzamide **289**.

According to General Procedure I, 3-nitro-phthalonitrile **238** (250.0 mg, 1.5 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 3 h. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 2-amino-6-cyano-benzoic acid **290** as an orange solid (23.0 mg, 10.0 %) and 2-amino-6-cyano-benzamide **289** as a yellow solid (116.0 mg, 49.7 %).

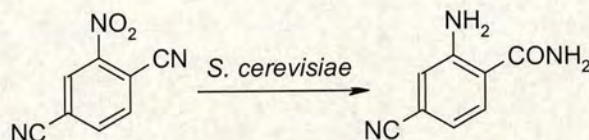
2-Amino-6-cyano-benzoic acid:  $R_f$  (EtOAc:Petrol, 1:1) 0.60; Mp 258.0-259.6 °C, (Found C, 59.28; H, 3.70; N, 17.26;  $C_8H_6N_2O_2$  requires C, 59.26; H, 3.73; N, 17.28);  $\nu_{max}(\text{KBr})/\text{cm}^{-1}$  3310, 3192 ( $\text{NH}_2$ ), 1750 ( $\text{C=O}$ ), 1719 ( $\text{NH}$ ), 1605 ( $\text{Ph}$ );  $\delta_H$  ( $(\text{CD}_3)_2\text{SO}$ , 360 MHz), 10.8 (H, br s,  $\text{COOH}$ ), 7.41 (1H, dd,  $J$  7.6 and 7.1,  $\text{PhH}$ ), 7.04 (1H, dd,  $J$  7.1 and 0.7,  $\text{PhH}$ ), 7.00 (1H, dd,  $J$  7.6 and 0.7,  $\text{PhH}$ ), 6.36 (2H, br s,  $\text{NH}_2$ );  $\delta_C$  ( $(\text{CD}_3)_2\text{SO}$ , 90 MHz) 171.1, 146.5 (C), 134.1 (CH), 133.5 (C), 121.1 (CH), 118.2 (C), 110.3 (CH);  $m/z$  (FAB) 163 ( $\text{MH}^+$ ), 135 (M-HCN), 117 (M-HCN- $\text{H}_2\text{O}$ ), Found ( $\text{MH}^+$ ) 163.06574,  $C_8H_6N_2O_2$  requires 163.0508; GC:  $R_t$  14.4 min, oven temp 150 °C.

2-Amino-6-cyano-benzamide:  $R_f$  (EtOAc:Petrol, 1:1) 0.13; Mp >350.0 °C;  $m/z$  (EI) 161 (M), 144 (M- $\text{NH}_3$ ), 117 (M- $\text{CONH}_2$ ); GC:  $R_t$  21.5 min, oven temp 150 °C; Data as in **289**.



**2-Amino-5-cyano-benzamide 291.**

According to General Procedure I, 4-nitro-isophthalonitrile **233** (250.0 mg, 1.5 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 30 min. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 2-amino-5-cyano-benzamide **291** as a yellow solid (145.0 mg, 62.3 %).  $R_f$  (EtOAc:Petrol, 1:1) 0.38; Mp 184.0-186.0 °C; (Found C, 59.31; H, 4.19; N, 25.77;  $C_8H_7N_3O$  requires C, 59.61; H, 4.38; N, 26.08);  $\nu_{max}$ (KBr)/ $cm^{-1}$  3409, 3346 ( $NH_2$ ), 3205 (CH), 2218 (CN), 1682 (C=O, NH), 1616 (Ph);  $\delta_H$  ( $(CD_3)_2CO$ , 200 MHz) 8.00 (1H, d,  $J$  1.6, PhH), 7.93 (1H, br s,  $CONH_2$ ), 7.45 (1H, dd,  $J$  8.5 and 1.6, PhH), 7.33 (1H, br s,  $CONH_2$ ), 6.77 (1H, d,  $J$  8.5, PhH), 3.17 (2H, br s,  $NH_2$ );  $\delta_C$  ( $(CD_3)_2CO$ , 63 MHz) 169.7, 153.7 (C), 134.7, 134.2 (CH), 120.5 (C), 116.9 (CH), 113.3, 95.1 (C);  $m/z$  ( $ES^+$ ) 162 ( $MH^+$ ), 144 ( $M-NH_3$ ), 117 ( $M-NH_3-HCN$ ), Found ( $MH^+$ ) 162.1707,  $C_8H_7N_3O$  requires 162.0670; GC:  $R_t$  14.2 min, oven temp 150 °C.

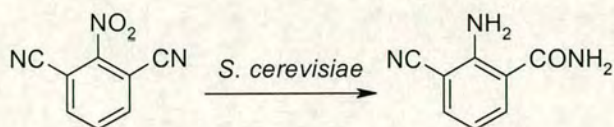
**2-Amino-4-cyano-benzamide 292.**

According to General Procedure I, 2-nitro-terephthalonitrile **222** (250.0 mg, 1.5 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 45 min. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 2-amino-4-cyano-benzamide **292** as a yellow solid (116.0 mg, 50.0 %).  $R_f$  (EtOAc) 0.54; Mp 201.0-202.5 °C, Lit.<sup>220</sup> 203.0-204.0 °C;  $\nu_{max}$ (KBr)/ $cm^{-1}$  3358, 3166 ( $NH_2$ ), 2109 (CH), 2232 (CN), 1674 (C=O), 1624 (NH), 1591 (Ph);  $\delta_H$  ( $(CD_3)_2SO$ , 200 MHz) 8.00 (1H, br s,  $CONH_2$ ), 7.66 (1H, d,  $J$  8.1, PhH), 7.41 (1H, br s,  $CONH_2$ ), 7.07 (1H, d,  $J$  1.7, PhH), 6.86 (1H, dd,  $J$  8.1 and 1.7, PhH), 6.84 (2H, br



s,  $\text{NH}_2$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 170.0, 150.0 (C), 130.0, 119.5 (CH), 118.9, 117.6 (C), 116.9 (CH), 114.0 (C);  $m/z$  (EI) 161 ( $\text{M}^+$ ), 144 ( $\text{M}-\text{NH}_3$ ), 117 ( $\text{M}-\text{NH}_3-\text{HCN}$ ); GC:  $R_t$  13.0 min, oven temp 150 °C.

### 2-Amino-3-cyano-benzamide **293**.



According to General Procedure I, 2-nitro-isophthalonitrile **230** (250.0 mg, 1.5 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 30 min. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 2-amino-3-cyano-benzamide **293** as a yellow solid (173.0 mg, 74.2 %).  $R_f$  (EtOAc:Petrol, 1:1) 0.46; Mp 222.0-223.7 °C, Lit.<sup>221</sup> 223.0-224.0 °C;  $\nu_{\text{max}}$ (KBr)/ $\text{cm}^{-1}$  3344, 3209 ( $\text{NH}_2$ ), 2219 (CN), 1694 (C=O), 1650 (NH), 1604 (Ph);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 8.03 (1H, br s,  $\text{CONH}_2$ ), 7.84 (1H, dd,  $J$  7.9 and 1.5, PhH), 7.61 (1H, dd,  $J$  7.8 and 1.5, PhH), 7.46 (1H, br s,  $\text{CONH}_2$ ), 7.22 (2H, br s,  $\text{NH}_2$ ), 6.64 (1H, dd,  $J$  7.9 and 7.8, PhH);  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 170.1, 151.8 (C), 136.5, 134.1 (CH), 117.6, 115.4 (C), 115.0 (CH), 96.5 (C);  $m/z$  (EI) 161 ( $\text{M}^+$ ), 144 ( $\text{M}-\text{NH}_3$ ), 117 ( $\text{M}-\text{NH}_3-\text{HCN}$ ); GC:  $R_t$  11.7 min, oven temp 150 °C.

### 4-Amino-phthalonitrile **295**.

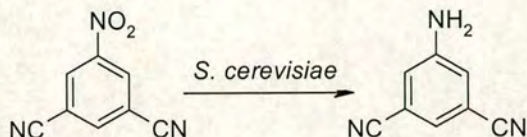


According to General Procedure I, 4-nitro-phthalonitrile **294** (250.0 mg, 1.5 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 2 h. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 4-amino-phthalonitrile **295** as an orange solid (96.0 mg, 46.2 %).



$R_f$  (EtOAc:Petrol, 1:1) 0.48; Mp 179.2-181.1 °C, Lit.<sup>222</sup> 180.0-181.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3292, 3367 (NH<sub>2</sub>), 3023 (CH), 2221 (CN), 1601 (Ph);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.78 (1H, dd,  $J$  8.7 and 1.5, PhH), 7.63 (1H, d,  $J$  8.7, PhH), 7.22 (1H, d,  $J$  1.5, PhH), 3.38 (2H, br s, NH<sub>2</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 154.8, 135.1 (C) 134.8 (CH), 117.3, 117.0, 115.5 (C), 115.1, 114.8 (CH);  $m/z$  (EI) 143 (M<sup>+</sup>), 116 (M-HCN), 100 (M-HCN-NH<sub>2</sub>); GC:  $R_t$  25.5 min, oven temp 120 °C.

#### 5-Amino-isophthalonitrile **296**.

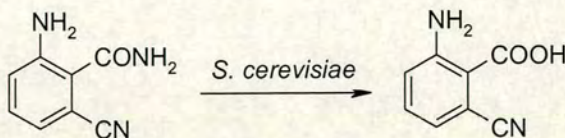


According to General Procedure I, 5-nitro-isophthalonitrile **221** (250.0 mg, 1.5 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 7 h. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 5-amino-isophthalonitrile **296** as a yellow solid (159.0 mg, 76.1 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.54; Mp 189.5-191.8 °C, Lit.<sup>223</sup> 192.0-193.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3333, 3192 (NH<sub>2</sub>), 2922 (CH), 2231 (CN), 1626 (NH), 1598 (Ph);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.38 (1H, t,  $J$  1.4, PhH), 7.15 (2H, d,  $J$  1.4, PhH), 6.14 (2H, br s, NH<sub>2</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 120.2, 119.1 (CH), 117.6, 113.2, 113.1 (C);  $m/z$  (EI) 143 (M<sup>+</sup>), 116 (M-HCN); GC:  $R_t$  11.7 min, oven temp 150 °C.

#### 7.4 Biotransformation : controls.

##### 2-Amino-6-cyano-benzoic acid **290**.

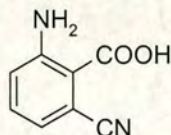


According to General Procedure I, 2-amino-6-cyano-benzamide **289** (48.3 mg, 0.3 mmol), *S. cerevisiae* (5.00 g) and water (20 mL) were incubated for 2 d. After



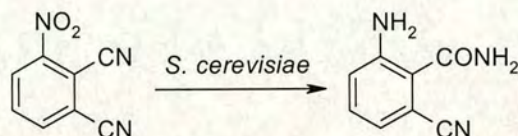
work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded *2-amino-6-cyano-benzoic acid* **290** as an orange solid (15.6 mg, 31.2 %).  $R_f$  (EtOAc:Petrol, 1:1) 0.58; Mp 257.1-258.9 °C; GC:  $R_t$  14.5 min, oven temp 150 °C; Data as in **290**.

### 2-Amino-6-cyano-benzoic acid **290**.



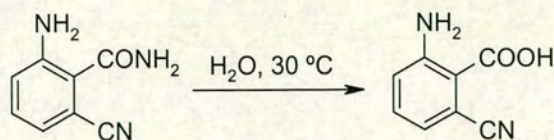
According to General Procedure I, 2-amino-6-cyano-benzoic acid **290** (48.6 mg, 0.3 mmol), *S. cerevisiae* (5.00 g) and water (20 mL) were incubated for 2 d. After work-up, purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded the *starting material* **290** as an orange solid (41.3 mg, 82.6 %).  $R_f$  (EtOAc:Petrol, 1:1) 0.59; Mp 258.0-259.6 °C; GC:  $R_t$  14.5 min, oven temp 150 °C; Data as in **290**.

### 2-Amino-6-cyano-benzamide **289** : Labelled $H_2^{18}O$



According to General Procedure I, 3-nitro-phthalonitrile **238** (6.2 mg, 3.6 mmol), *S. cerevisiae* (62.5 mg) and  $^{18}O$ -labelled water (250.0  $\mu$ L) were incubated for 2 d. (The small-scale biotransformation was carried out in an eppendorf tube). Ethyl acetate was added and the biphasic layers were mixed using a rotameter and microcentrifuged. The top organic layer was removed and subjected to GCMS analysis which indicated the presence of *2-amino-6-cyano-benzamide* **239**.  $m/z$  (EI) 161 ( $M^+$ ), 144 ( $M-NH_3$ ), 117 ( $M-CONH_2$ ); GC:  $R_t$  21.8 min, oven temp 150 °C.



**2-Amino-6-cyano-benzoic acid 290.**

2-Amino-6-cyano-benzamide **289** (48.3 mg, 0.3 mmol) was added to water (20 mL) and incubated in the orbital shaker at 30 °C and 200 rpm. The hydrolysis of the substrate was monitored by GCMS of 1 mL samples withdrawn at regular time intervals from the reaction mixture. Each sample was added to ethyl acetate (1 mL) in an eppendorf tube and the resulting biphasic solution was mixed using a rotameter and then microcentrifuged to separate the layers. The organic layer (800  $\mu$ L) containing the product(s) was removed and further diluted with ethyl acetate (500  $\mu$ L) and subjected to GCMS analysis. After 2 d, the aqueous solution was extracted with chloroform (2 x 25 mL) and the combined organic extracts were dried ( $\text{MgSO}_4$ ), filtered and concentrated under reduced pressure to yield a solid. Purification by flash-column chromatography (ethyl acetate:petrol, 1:1) afforded 2-amino-6-cyano-benzoic acid **290** as an orange solid (13.8 mg, 28.4 %).

$R_f$  (EtOAc:Petrol, 1:1) 0.59; Mp 256.6-257.8 °C; GC:  $R_t$  14.5 min, oven temp 150 °C; Data as in **290**.



## 7.5 General Procedure II : *S. cerevisiae* catalysed reduction of (di)nitroarene-sulfinyls.

A solution of the substrate (250.0 mg) in the minimum amount of dioxane was added to a suspension of *S. cerevisiae* (25.00 g) in water (100 mL) and incubated. The reduction of the substrate was monitored by chiral HPLC (hexane:*iso*-propanol/EtOH) of 1 mL samples withdrawn at regular time intervals from the reaction mixture. Each sample was microcentrifuged, 400  $\mu$ L of the aqueous layer was removed and filtered before being diluted with 600  $\mu$ L of mobile phase and subjected to chiral HPLC analysis.

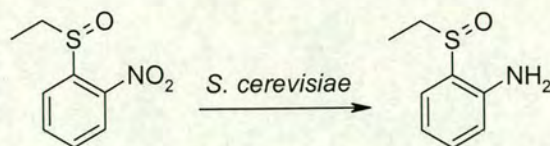
Upon complete substrate consumption, the *S. cerevisiae* suspension was continuously extracted with chloroform overnight. The organic extract was dried ( $\text{MgSO}_4$ ), filtered and concentrated *in vacuo* to yield the crude product. Further purification of the crude was achieved by flash-column chromatography.

Reaction times, purification methods and yields are given for each individual biotransformation.



## 7.5.1 Reduction of nitroarene-sulfinyls.

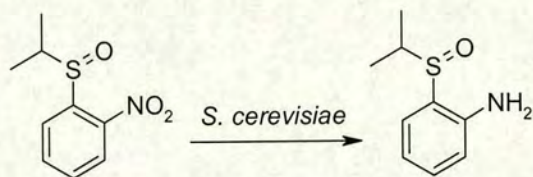
## 2-Ethanesulfinyl-phenylamine 302.



According to General Procedure II, ethanesulfinyl-2-nitrobenzene **261** (250.0 mg, 1.3 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 6 h. After work-up, purification by flash-column chromatography (chloroform:ethanol, 9:1) afforded 2-ethanesulfinyl-phenylamine **302** as a yellow solid (136.0 mg, 61.9 %).

$R_f$  (CHCl<sub>3</sub>:MeOH, 9:1) 0.47; Mp 110.3-112.1 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3087 (CH), 3335, 3134 (NH<sub>2</sub>), 1602 (Ph), 1081 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.35 (2H, m, PhH), 7.22 (1H, td,  $J$  8.0, 8.2 and 1.1, PhH), 6.71 (1H, dd,  $J$  8.0 and 1.1, PhH), 5.00 (2H, br s, NH<sub>2</sub>), 3.68 (1H, q,  $J$  7.1, CH<sub>2</sub>), 2.80 (1H, q,  $J$  7.5, CH<sub>2</sub>), 1.35 (1H, t,  $J$  7.5 and 7.1, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 144.1, 140.3 (C), 133.2, 127.8, 122.2, 118.0 (CH), 45.1 (CH<sub>2</sub>), 9.2 (CH<sub>3</sub>);  $m/z$  (EI) 169 (M<sup>+</sup>), 140 (M-C<sub>2</sub>H<sub>5</sub>), 92 (M-C<sub>2</sub>H<sub>5</sub>SO), 65 (M-C<sub>2</sub>H<sub>5</sub>SO-HCN), Found (M<sup>+</sup>) 169.0496, C<sub>8</sub>H<sub>11</sub>NSO requires 169.0561; HPLC (hexane:EtOH, 90:10):  $R_t$  18.6 and 20.9 min.

## 2-(Propane-2-sulfinyl)-phenylamine 303.

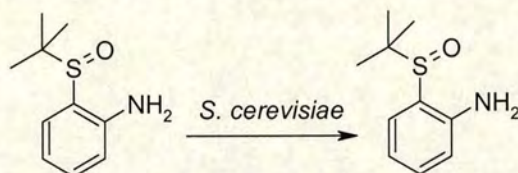


According to General Procedure II, 1-nitro-2-(propane-2-sulfinyl)-benzene **262** (255.6 mg, 1.2 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 6 h. After work-up, purification by flash-column chromatography (chloroform:methanol, 9:1) afforded 2-(propane-2-sulfinyl)-phenylamine **303** as a yellow solid (98.0 mg, 45.2 %).



$R_f$  ( $\text{CHCl}_3$ :MeOH, 1:1) 0.33; Mp 9105.3-106.9 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3310, 3112 ( $\text{NH}_2$ ), 2991 (CH), 1584 (Ph), 1055 (S=O);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 7.31 (1H, dd,  $J$  8.2 and 1.0, PhH), 7.25 (1H, td,  $J$  8.2, 8.0 and 1.2, PhH), 7.20 (1H, td,  $J$  8.0, 7.8 and 1.0, PhH), 6.87 (1H, dd,  $J$  7.8 and 1.2, PhH), 4.67 (2H, br s,  $\text{NH}_2$ ), 3.17 (1H, sept,  $J$  6.8, CH), 1.53 (3H, d,  $J$  6.8,  $\text{CH}_3$ ), 0.91 (3H, d,  $J$  6.8,  $\text{CH}_3$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 142.8 (C), 132.2, 129.3 (CH), 128.2 (C), 124.1, 120.8, 50.9 (CH), 17.1, 15.2 ( $\text{CH}_3$ );  $m/z$  (EI) 183 ( $\text{M}^+$ ), 140 ( $\text{M}-\text{C}_3\text{H}_7$ ), 92 ( $\text{M}-\text{C}_3\text{H}_7-\text{SO}$ ), Found ( $\text{M}^+$ ) 183.0701,  $\text{C}_9\text{H}_{13}\text{NSO}$  requires 183.0718; HPLC (hexane:EtOH, 99:1):  $R_t$  35.8 and 43.2 min.

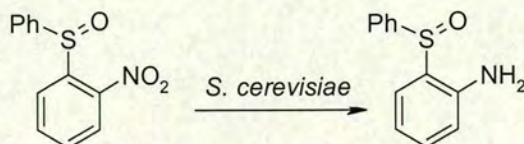
### 2-(2-Methyl-propane-2-sulfinyl)-phenylamine 304.



According to General Procedure II, butylsulfinyl-2-nitrobenzene **263** (227.0 mg, 1.0 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 5 h. After work-up, purification by flash-column chromatography (chloroform:methanol, 9:1) afforded methyl-propane-2-sulfinyl)-phenylamine **304** as a yellow solid (112.0 mg, 49.3 %).

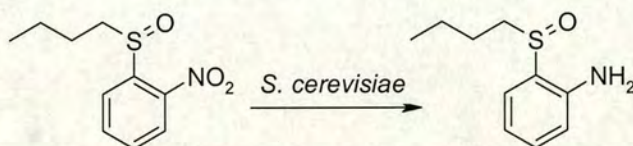
$R_f$  ( $\text{CHCl}_3$ :MeOH, 9:1) 0.37; Mp 121.3-122.7 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3356, 3140, ( $\text{NH}_2$ ), 3015 (CH), 1605 (Ph), 1057 (S=O);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 7.79 (1H, dd,  $J$  8.0, 0.9, PhH), 7.62 (1H, td,  $J$  8.0, 7.8 and 1.2, PhH), 7.52 (1H, td,  $J$  7.9, 7.8 and 0.9, PhH), 7.31 (1H, dd,  $J$  7.9 and 1.2, PhH), 4.85 (2H, br s,  $\text{NH}_2$ ), 1.40 (3H, s,  $\text{CH}_3$ ), 1.40 (3H, s,  $\text{CH}_3$ ), 1.27 (3H, s,  $\text{CH}_3$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 148.2, 140.8 (C), 134.7, 128.1, 120.9, 118.7 (CH), 50.1 (C), 25.2 ( $\text{CH}_3$ );  $m/z$  (EI) 197 ( $\text{M}^+$ ), 140 ( $\text{M}-\text{C}(\text{CH}_3)_3$ ), 92 ( $\text{M}-\text{SOC}(\text{CH}_3)_3$ ), Found ( $\text{M}^+$ ) 197.0796,  $\text{C}_{10}\text{H}_{15}\text{NSO}$  requires 197.0874; HPLC (hexane:EtOH, 99:1):  $R_t$  40.1 and 46.2 min.



**2-Benzenesulfinyl-phenylamine 305.**

According to General Procedure II, 1-(benzenesulfinyl)-2-nitrobenzene **264** (247.0 mg, 1.0 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 5 d. After work-up, purification by flash-column chromatography (chloroform:methanol, 9:1) afforded 2-benzenesulfinyl-phenylamine **305** as a yellow solid (98.0 mg, 45.2 %).

$R_f$  (CHCl<sub>3</sub>:MeOH, 9:1) 0.39; Mp 95.0-96.9 °C, Lit.<sup>224</sup> 97.5-98.5 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3298, 3084 (NH<sub>2</sub>), 2977 (CH), 1610 (Ph), 1055 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.56 (2H, m, Ph'H), 7.42 (4H, m, Ph'H and PhH), 7.28 (1H, td, *J* 8.4 and 0.7, PhH), 6.74 (1H, td, *J* 8.4, 8.1 and 1.1, PhH), 6.56 (1H, dd, *J* 8.1 and 0.7, PhH), 4.7 (2H, br s, NH<sub>2</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 147.5, 143.1 (C), 132.8, 130.1, 128.6, 128.2, 124.6 (CH), 120.3 (C), 117.4, 116.9 (CH); *m/z* (EI) 217 (M<sup>+</sup>), 187 (M-HCN), 110 (M-HCN-C<sub>6</sub>H<sub>5</sub>); HPLC (hexane:*iso*-propanol, 95:5):  $R_t$  54.8 and 71.7 min.

**2-(Butane-1-sulfinyl)-phenylamine 306.**

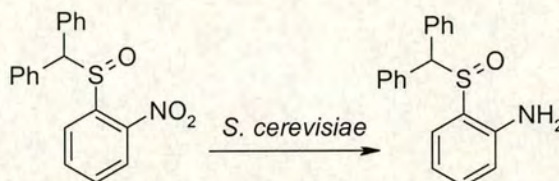
According to General Procedure II, 1-(butane-1-sulfinyl)-2-nitrobenzene **265** (250.0 mg, 1.1 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 7 h. After work-up, purification by flash-column chromatography (chloroform:ethanol, 9:1) afforded 2-(butane-1-sulfinyl)-phenylamine **306** as a yellow solid (120.0 mg, 55.2 %).

$R_f$  (CHCl<sub>3</sub>:MeOH, 9:1) 0.38; Mp 73.0-75.8°C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3358, 3162, (NH<sub>2</sub>), 3011 (CH), 1587 (Ph), 1055 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.42 (1H, dd, *J* 7.9 and 1.2, PhH), 7.31 (2H, m, PhH), 7.27 (1H, td, *J* 7.9 and 1.0, PhH), 4.80 (2H, br s,



$\text{NH}_2$ ), 3.10 (2H, m,  $\text{S(O)CH}_2$ ), 1.75 (4H, m,  $\text{CH}_2\text{CH}_2\text{CH}_3$ ), 0.94 (3H, t,  $J$  5.2,  $\text{CH}_3$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 145.3, 136.1 (C), 135.8, 130.2, 121.4, 119.3 (CH), 53.1, 37.0, 30.8 ( $\text{CH}_2$ ), 11.1 ( $\text{CH}_3$ );  $m/z$  (EI) 197 ( $\text{M}^+$ ), 140 (M- $\text{C}_4\text{H}_9$ ), 92 (M- $\text{C}_4\text{H}_9\text{-SO}$ ), Found ( $\text{M}^+$ ) 197.0839,  $\text{C}_{10}\text{H}_{15}\text{NSO}$  requires 197.0874; HPLC (hexane:EtOH, 90:10):  $R_t$  24.9 and 32.1 min.

### 2-(Diphenyl-methanesulfinyl)-phenylamine 307.



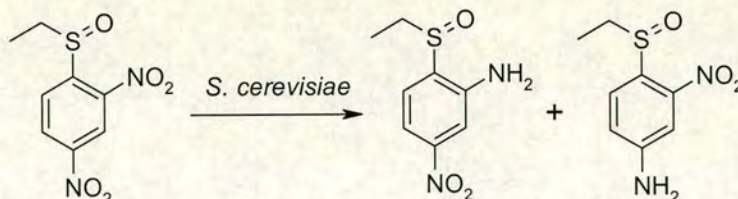
According to General Procedure II, 1-(diphenyl-methanesulfinyl)-2-nitrobenzene **266** (235.9 mg, 0.7 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 24 h. After work-up, purification by flash-column chromatography (chloroform:methanol, 9:1) afforded 2-(diphenyl-methanesulfinyl)-phenylamine **307** as a yellow solid (122.0 mg, 56.2 %).

$R_f$  ( $\text{CHCl}_3$ :MeOH) 0.31; Mp 132.3-133.8 °C;  $\nu_{\text{max}}$ (KBr)/ $\text{cm}^{-1}$  3385, 3175 ( $\text{NH}_2$ ), 2974 (CH), 1592 (Ph), 1084 (S=O);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 8.58 (1H, dd,  $J$  7.8 and 1.4, PhH), 8.25 (4H, m, Ph'H), 8.02 (4H, m, Ph'H), 7.71 (2H, m, Ph'H), 7.01 (2H, m, PhH), 6.65 (1H, dd,  $J$  8.1 and 1.2, PhH), 5.22 (1H, s, CH);  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 145.0, 143.7, 136.8 (C), 135.3, 131.4, 131.3, 129.1, 126.6, 126.2, 125.2, 120.1, 60.2 (CH);  $m/z$  (EI) 307 ( $\text{M}^+$ ), 108 (M-SCH( $\text{C}_6\text{H}_5$ )<sub>2</sub>), 178 (M-SCH( $\text{C}_6\text{H}_5$ )<sub>2</sub>-NO), Found ( $\text{M}^+$ ) 307.1009,  $\text{C}_{10}\text{H}_{15}\text{NSO}$  requires 307.1031; HPLC (hexane:EtOH, 60:40):  $R_t$  30.8 and 38.2 min.



## 7.5.2 Reduction of dinitroarene-sulfinyls.

2-Ethanesulfinyl-5-nitro-phenylamine 310 and 4-Ethanesulfinyl-3-nitro-phenylamine 311.



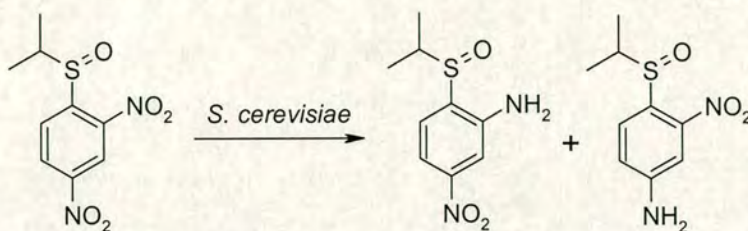
According to General Procedure II, 1-ethanesulfinyl-2,4-dinitrobenzene **275** (244.0 mg, 1.0 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 4 h. After work-up, purification by flash-column chromatography (chloroform:methanol, 8:2) afforded 2-ethanesulfinyl-5-nitro-phenylamine **310** as a yellow solid (71.0 mg, 33.2 %) and 4-ethanesulfinyl-3-nitro-phenylamine **311** as an orange gum (65.2 mg, 30.5 %).

2-Ethanesulfinyl-5-nitro-phenylamine:  $R_f$  (CHCl<sub>3</sub>:MeOH, 8:2) 0.45; Mp 122.3-124.9 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3352, 3142 (NH<sub>2</sub>), 2987 (CH), 1612 (Ph), 1530, 1334 (NO<sub>2</sub>), 1024 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.68 (1H, dd,  $J$  8.2 and 1.2, PhH), 7.35 (1H, d,  $J$  8.2, PhH), 7.12 (1H, d,  $J$  1.3, PhH), 3.31 (1H, q,  $J$  7.1, CH<sub>2</sub>), 2.80 (1H, q,  $J$  7.1, CH<sub>2</sub>), 1.24 (3H, t,  $J$  7.1, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 148.2, 146.3, 138.1 (C), 127.2, 124.7, 120.2 (CH), 47.1 (CH<sub>2</sub>), 10.1 (CH<sub>3</sub>);  $m/z$  (EI) 214 (M<sup>+</sup>), 185 (M-C<sub>2</sub>H<sub>5</sub>), 137 (M-SOC<sub>2</sub>H<sub>5</sub>), 110 (M-SOC<sub>2</sub>H<sub>5</sub>-NH<sub>2</sub>), Found (M<sup>+</sup>) 214.0426, C<sub>8</sub>H<sub>10</sub>N<sub>2</sub>SO<sub>3</sub> requires 214.0412; HPLC (hexane:EtOH, 80:20):  $R_t$  10.6 min.

4-Ethanesulfinyl-3-nitro-phenylamine:  $R_f$  (CHCl<sub>3</sub>:MeOH, 8:2) 0.39;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3361, 3145 (NH<sub>2</sub>), 3010 (CH), 1615 (Ph), 1541, 1341 (NO<sub>2</sub>), 1030 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.90 (1H, d,  $J$  8.2, PhH), 7.21 (1H, d,  $J$  1.2, PhH), 6.99 (1H, dd,  $J$  8.2 and 1.2, PhH), 3.28 (1H, q,  $J$  6.8, CH<sub>2</sub>), 7.76 (1H, q,  $J$  6.8, CH<sub>2</sub>), 1.21 (3H, t,  $J$  6.8, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 147.1, 145.1, 130.0 (C), 125.9, 123.2, 119.5 (CH), 45.7 (CH<sub>2</sub>), 9.2 (CH<sub>3</sub>);  $m/z$  (EI) 214 (M<sup>+</sup>), 137 (M-SOC<sub>2</sub>H<sub>5</sub>), 110 (M-SOC<sub>2</sub>H<sub>5</sub>-NH<sub>2</sub>), Found (M<sup>+</sup>) 214.0410, C<sub>8</sub>H<sub>10</sub>N<sub>2</sub>SO<sub>3</sub> requires 214.0412; HPLC (hexane:EtOH, 80:20):  $R_t$  11.8 min.



**2-(Propane-2-sulfinyl)-5-nitro-phenylamine 312 and 4-(Propane-2-sulfinyl)-3-nitro-phenylamine 313.**



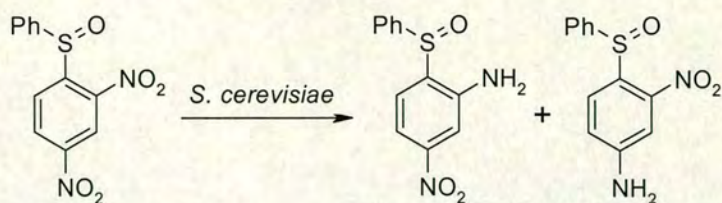
According to General Procedure II, 1-(propane-2-sulfinyl)-2,4-dinitrobenzene **276** (258.0 mg, 1.0 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 3 h. After work-up, purification by flash-column chromatography (chloroform:methanol, 9:1) afforded 2-(propane-2-sulfinyl)-5-nitro-phenylamine **312** as a yellow solid (69.3 mg, 30.4 %) and 4-(propane-2-sulfinyl)-3-nitro-phenylamine **313** as a yellow gum (66.6 mg, 29.2 %).

2-(Propane-2-sulfinyl)-5-nitro-phenylamine:  $R_f$  (CHCl<sub>3</sub>:MeOH, 9:1) 0.39 ; Mp 122.3-124.1 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3295, 3087 (NH<sub>2</sub>), 3011 (CH), 1615 (Ph), 1530, 1338 (NO<sub>2</sub>), 1050 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.34 (1H, dd,  $J$  8.5 and 1.6, PhH), 8.01 (1H, d,  $J$  8.5, PhH), 7.84 (1H, d,  $J$  1.6, PhH), 3.20 (1H, q,  $J$  7.0, CH), 1.57 (3H, d,  $J$  7.0, CH<sub>3</sub>), 0.97 (3H, d,  $J$  7.0, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 149.2, 141.2, 136.2 (C), 124.1, 120.1, 115.2, 51.4 (CH), 18.1, 15.2 (CH<sub>3</sub>);  $m/z$  (EI) 228 (M<sup>+</sup>), 121 (M-SOC<sub>3</sub>H<sub>7</sub>), 91 (M-SOC<sub>3</sub>H<sub>7</sub>-NO), Found (M<sup>+</sup>) 228.0489, C<sub>9</sub>H<sub>12</sub>N<sub>2</sub>SO<sub>3</sub> requires 228.0569; HPLC (hexane:EtOH, 60:40):  $R_t$  10.0 min.

4-(Propane-2-sulfinyl)-3-nitro-phenylamine:  $R_f$  (CHCl<sub>3</sub>:MeOH, 9:1) 0.32;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3285, 3102 (NH<sub>2</sub>), 3000 (CH), 1605 (Ph), 1541, 1342 (NO<sub>2</sub>), 1051 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.14 (1H, d,  $J$  8.1, PhH), 7.93 (1H, d,  $J$  1.9, PhH), 7.68 (1H, dd,  $J$  8.1 and 1.9, PhH), 3.17 (1H, q,  $J$  6.8, CH), 1.51 (3H, d,  $J$  6.8, CH<sub>3</sub>), 0.87 (6H, d,  $J$  6.8, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 148.2, 139.5, 129.5 (C), 123.5, 122.1, 113.4, 50.3 (CH), 14.7, 12.5 (CH<sub>3</sub>);  $m/z$  (EI) 228 (M<sup>+</sup>), 91 (M-SOC<sub>3</sub>H<sub>7</sub>-NO), Found (M<sup>+</sup>) 228.0510, C<sub>9</sub>H<sub>12</sub>N<sub>2</sub>SO<sub>3</sub> requires 228.0569; HPLC (hexane:EtOH, 60:40):  $R_t$  11.8 min.



**2-Benzenesulfinyl-5-nitro-phenylamine 316 and 4-Benzenesulfinyl-3-nitro-phenylamine 317.**



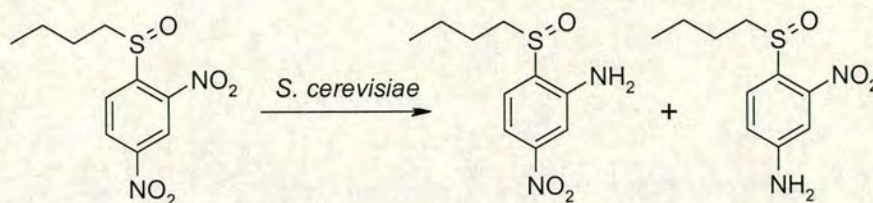
According to General Procedure II, 1-benzenesulfinyl-2,4-dinitrobenzene **277** (234.0 mg, 0.9 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 2 h. After work-up, purification by flash-column chromatography (chloroform:methanol, 8:2) afforded 2-benzenesulfinyl-5-nitro-phenylamine **316** as a yellow solid (84.0 mg, 35.6 %) and 4-benzenesulfinyl-3-nitro-phenylamine **317** as a yellow solid (75.7 mg, 32.1 %).

2-Benzenesulfinyl-5-nitro-phenylamine:  $R_f$  ( $\text{CHCl}_3$ :MeOH, 8:2) 0.31; 132.3-133.9 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3521, 3124 ( $\text{NH}_2$ ), 3098 (CH), 1598 (Ph), 1551, 1362 ( $\text{NO}_2$ ), 1069 (S=O);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 8.45 (1H, dd,  $J$  8.0 and 1.8, PhH), 8.20 (1H, d,  $J$  8.0, PhH), 8.18 (6H, m, PhH and Ph'H);  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 148.2, 146.1 (C), 130.1, 128.7, 128.1 (CH), 127.8, 126.3 (C), 124.2, 122.1, 121.9, 118.2, 115.1 (CH);  $m/z$  (EI) 262 ( $\text{M}^+$ ), 185 (M-C<sub>6</sub>H<sub>5</sub>), 137 (M-SOC<sub>6</sub>H<sub>5</sub>), Found ( $\text{M}^+$ ) 262.0400, C<sub>12</sub>H<sub>10</sub>N<sub>2</sub>SO<sub>3</sub> requires 262.0412; HPLC (hexane:EtOH, 60:40):  $R_t$  6.1 min.

4-Benzenesulfinyl-3-nitro-phenylamine:  $R_f$  ( $\text{CHCl}_3$ :MeOH, 8:2) 0.28; Mp 119.2-120.1 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3532, 3130 ( $\text{NH}_2$ ), 3054 (CH), 1599 (Ph), 1541, 1348 ( $\text{NO}_2$ ), 1055 (S=O);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 8.01 (1H, d,  $J$  8.8, PhH), 7.50 (6H, m, PhH and Ph'H), 7.22 (1H, dd,  $J$  8.8 and 1.1, PhH);  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 148.1, 144.1 (C), 131.5, 129.6 (CH), 129.8 (C), 127.5, 126.1, 125.7, 122.4 (CH), 121.8 (C), 118.2 (CH);  $m/z$  (EI) 262 ( $\text{M}^+$ ), 137 (M-SOC<sub>6</sub>H<sub>5</sub>), 107 (M-SOC<sub>6</sub>H<sub>5</sub>-NO), Found ( $\text{M}^+$ ) 262.0405, C<sub>12</sub>H<sub>10</sub>N<sub>2</sub>SO<sub>3</sub> requires 262.0412; HPLC (hexane:EtOH, 60:40):  $R_t$  18.2 min.



**2-(Butane-1-sulfinyl)-5-nitro-phenylamine 314 and 4-(Butane-1-sulfinyl)-3-nitro-phenylamine 315.**



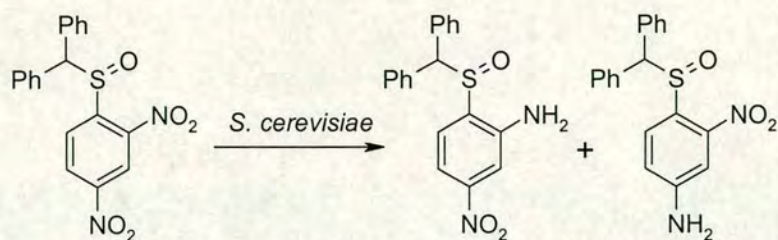
According to General Procedure II, 1-(butane-1-sulfinyl)-2,4-dinitrobenzene **278** (244.8 mg, 0.9 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 4 h. After work-up, purification by flash-column chromatography (chloroform:methanol, 9:1) afforded 2-(butane-1-sulfinyl)-5-nitro phenylamine **314** as a yellow solid (80.4 mg, 36.9 %) and 4-(butane-1-sulfinyl)-3-nitro-phenylamine **315** as an orange oil (67.8 mg, 31.2 %).

2-(Butane-1-sulfinyl)-5-nitro phenylamine:  $R_f$  (CHCl<sub>3</sub>:MeOH, 9:1) 0.34; Mp 108.9–111.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3074 (CH), 1602 (Ph), 1524, 1335 (NO<sub>2</sub>), 1026 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.25 (1H, dd,  $J$  7.6 and 1.5, PhH), 8.01 (1H, d,  $J$  7.6, PhH), 7.76 (1H, d,  $J$  1.5, PhH), 7.76 (1H, d,  $J$  7.6, PhH), 3.35 (1H, t, SCH<sub>2</sub>), 3.30 (1H, t, SCH<sub>2</sub>), 1.72 (4H, m, CH<sub>2</sub>CH<sub>2</sub>Me), 1.42 (3H, t, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 149.7, 141.6, 136.8 (C), 126.4, 124.1, 118.2 (CH), 47.3, 30.5, 22.5 (CH<sub>2</sub>), 14.1 (CH<sub>3</sub>);  $m/z$  (EI) 242 (M<sup>+</sup>), 185 (M-C<sub>4</sub>H<sub>9</sub>), 137 (M-SOC<sub>4</sub>H<sub>9</sub>), 91 (M-SC<sub>4</sub>H<sub>9</sub>-NO<sub>2</sub>), Found (M<sup>+</sup>) 242.0765, C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub>S requires 242.0725; HPLC (hexane:EtOH, 75:25):  $R_t$  5.2 min.

4-(Butane-1-sulfinyl)-3-nitro-phenylamine:  $R_f$  (CHCl<sub>3</sub>:MeOH, 9:1) 0.30;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3061 (CH), 1598 (Ph), 1528, 1336 (NO<sub>2</sub>), 1027 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.17 (1H, d,  $J$  7.9, PhH), 7.89 (1H, d,  $J$  1.8, PhH), 7.65 (1H, dd,  $J$  7.9 and 1.8, PhH), 3.29 (1H, t, SCH<sub>2</sub>), 3.22 (1H, t, SCH<sub>2</sub>), 1.65 (4H, m, CH<sub>2</sub>CH<sub>2</sub>Me), 1.33 (3H, t, CH<sub>3</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 148.5, 141.2, 135.0 (C), 127.1, 123.4, 118.1 (CH), 48.5, 28.7, 21.4 (CH<sub>2</sub>), 13.9 (CH<sub>3</sub>);  $m/z$  (EI) 242 (M<sup>+</sup>), 185 (M-C<sub>4</sub>H<sub>9</sub>), 137 (M-SOC<sub>4</sub>H<sub>9</sub>), 91 (M-SC<sub>4</sub>H<sub>9</sub>-NO<sub>2</sub>), Found (M<sup>+</sup>) 242.0712, C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub>S requires 242.0725; HPLC (hexane:EtOH, 75:25):  $R_t$  12.8 min.



**2-(Diphenyl-methane-sulfinyl)-5-nitro-phenylamine 318 and 4-(Diphenyl-methane-sulfinyl)-3-nitro-phenylamine 319.**



According to General Procedure II, 1-(diphenyl-methane-sulfinyl)-2,4-dinitrobenzene **281** (245.0 mg, 0.7 mmol), *S. cerevisiae* (25.00 g) and water (100 mL) were incubated for 3 h. After work-up, purification by flash-column chromatography (chloroform:methanol, 9:1) afforded 2-(diphenyl-methane-sulfinyl)-5-nitro-phenylamine **318** as a yellow solid (82.2 mg, 33.4 %) and 4-(diphenyl-methane-sulfinyl)-3-nitro-phenylamine **319** as a yellow solid (76.1 mg, 30.9 %).

2-(Diphenyl-methane-sulfinyl)-5-nitro-phenylamine:  $R_f$  (CHCl<sub>3</sub>:MeOH, 9:1) 0.31; Mp 142.3-143.8 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3318, 3101 (NH<sub>2</sub>), 3068 (CH), 1585 (Ph), 1541, 1351 (NO<sub>2</sub>), 1068 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.49 (1H, dd,  $J$  8.0 and 1.9, PhH), 8.05 (1H, d,  $J$  8.0, PhH), 7.81 (9H, m, PhH and Ph'H), 7.77 (2H, m, Ph'H), 4.91 (1H, s, CH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 149.2, 145.1, 139.9, 137.1 (C), 132.9, 130.7 (CH), 129.8, 128.7 (C), 128.3, 125.4, 124.2, 120.2, 119.9, 118.3, 115.2 (CH);  $m/z$  (EI) 352 (M<sup>+</sup>), 137 (M-SOCH(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub>), 107 (M-SOCH(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub>-NO), 91 (M-SOCH(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub>-NO-NH<sub>2</sub>), Found (M<sup>+</sup>) 352.0910, C<sub>9</sub>H<sub>12</sub>N<sub>2</sub>SO<sub>3</sub> requires 352.0882; HPLC (hexane:EtOH, 50:50):  $R_t$  6.2 min.

4-(Diphenyl-methane-sulfinyl)-3-nitro-phenylamine:  $R_f$  (CHCl<sub>3</sub>:MeOH, 9:1) 0.27; Mp 132.7-133.1 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3304, 33298 (NH<sub>2</sub>), 3055 (CH), 1581 (Ph), 1530, 1348 (NO<sub>2</sub>), 1041 (S=O);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.26 (1H, d,  $J$  7.7, PhH), 7.91 (1H, d,  $J$  1.4, PhH), 7.52 (8H, m, Ph'H), 7.40 (2H, m, Ph'H), 7.00 (1H, dd,  $J$  7.7 and 1.4, PhH), 4.87 (1H, s, CH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 144.2, 135.1, 131.1 (C), 133.8, 131.4, 128.2, 125.1 (CH), 120.8 (C), 119.8, 118.1, 117.2, 111.1 (CH);  $m/z$  (EI) 352 (M<sup>+</sup>), 137 (M-SOCH(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub>), 107 (M-SCH(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub>-NO), Found (M<sup>+</sup>) 352.0879, C<sub>9</sub>H<sub>12</sub>N<sub>2</sub>SO<sub>3</sub> requires 352.0882; HPLC (hexane:EtOH, 50:50):  $R_t$  6.9 min.

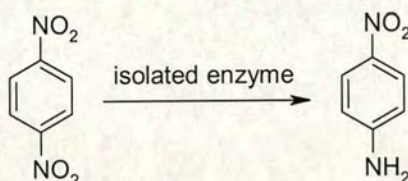


## 7.6 General Procedure III : Nitro-Reductase Studies : Qualitative Studies.

The enzyme (10  $\mu$ L, 2.0 mmol) and a cofactor, either NADH or NADPH (50  $\mu$ L, 10.0 mmol) were added to tris buffer (pH 8, 900  $\mu$ L, 50.0 mmol) in an eppendorf tube and incubated in an orbital shaker at 30 °C and 200 rpm for 30 min. The substrate (10  $\mu$ L, 50.0 mmol) in the minimum amount of DMSO was added to the enzyme/buffer mixture. The reduction of the substrate was monitored by GCMS of 500  $\mu$ L samples withdrawn at regular time intervals from the reaction mixture. Each sample was added to ethyl acetate (500  $\mu$ L) in an eppendorf tube and the resulting biphasic solution was mixed using a rotameter, then microcentrifuged to separate the layers. The organic layer (400  $\mu$ L) was removed and further diluted with ethyl acetate (200  $\mu$ L) and subjected to GCMS analysis.

The results are measured in percentage conversion to product with respect to starting material over 1 h and 2 h time periods.

### 7.6.1 4-Nitro-aniline **321**.



According to General Procedure III, 1,4-dinitrobenzene **320** was used as a substrate, with the enzyme or cofactor varied with each reaction as indicated in bold.

1. 1,4-Dinitrobenzene, **NRase I**, **NADPH** and buffer were incubated. GCMS analysis (after 1 h) indicated the presence of *4-nitro-aniline* **321**.

Product conversion: 28.1 % (1 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO), 76 (M-NO-HCN).

1i. 1,4-Dinitrobenzene, **NRase I**, **NADH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *4-nitro-aniline* **321**.



Product conversion: 12.9 % (1 h), 31.1 % (2 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO), 76 (M-NO-HCN).

2. 1,4-Dinitrobenzene, **NRase II**, **NADPH** and buffer were incubated. GCMS analysis (after 1 h) indicated the presence of *4-nitro-aniline* **321**.

Product conversion: 24.1 % (1 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO), 76 (M-NO-HCN).

2i. 1,4-Dinitrobenzene, **NRase II**, **NADH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *4-nitro-aniline* **321**.

Product conversion: 6.5 % (1 h), 11.2 % (2 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO), 76 (M-NO-HCN).

3. 1,4-Dinitrobenzene, **NRase III**, **NADPH** and buffer were incubated. GCMS analysis (after 1 h) indicated the presence of *4-nitro-aniline* **321**.

Product conversion: 32.1 % (1 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO), 76 (M-NO-HCN).

3i. 1,4-Dinitrobenzene, **NRase III**, **NADH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *4-nitro-aniline* **321**.

Product conversion: 11.2 % (1 h), 30.6 % (2 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO).

4. 1,4-Dinitrobenzene, **glutathione reductase**, **NADPH** and buffer were incubated. GCMS analysis indicated the presence of the *starting material* **320**.

4i. 1,4-Dinitrobenzene, **glutathione reductase**, **NADH** and buffer were incubated. GCMS analysis indicated the presence of the *starting material* **320**.

5. 1,4-Dinitrobenzene, **L-lactate dehydrogenase**, **NADPH** and buffer were incubated. GCMS analysis (after 1h) indicated the presence of *4-nitro-aniline* **321**.



Product conversion: 10.0 % (1 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO), 76 (M-NO-HCN).

5i. 1,4-Dinitrobenzene, **L-lactate dehydrogenase**, **NADH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *4-nitro-aniline* **321**.

Product conversion: 24.8 % (1 h), 6.2 % (2 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO), 76 (M-NO-HCN).

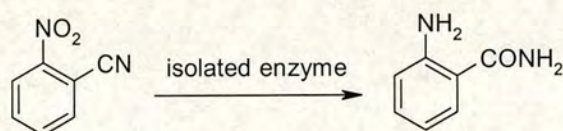
6. 1,4-Dinitrobenzene, P-450 **Cytochrome reductase**, **NADPH** and buffer were incubated. GCMS analysis (after 1 h) indicated the presence of *4-nitro-aniline* **321**.

Product conversion: 35.1 % (1 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO), 76 (M-NO-HCN).

6i. 1,4-Dinitrobenzene, P-450 **Cytochrome reductase**, **NADH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *4-nitro-aniline* **321**.

Product conversion: 8.9 % (1 h), 6.0 % (2 h);  $R_t$  3.1 min, oven temp 150 °C;  $m/z$  (EI) 138 ( $M^+$ ), 103 (M-NO), 76 (M-NO-HCN).

#### 7.6.2 2-Amino-benzamide **284**.



According to General Procedure III, 2-nitro-benzonitrile **282** was used as a substrate, with the enzyme or cofactor varied with each reaction as indicated in bold.

1ii. 2-Nitro-benzonitrile, **NRase I**, **NADPH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *2-amino-benzamide* **284**.

Product conversion: 35.6 % (1 h), 66.2 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65 (M-CONH<sub>2</sub>-HCN).



1iii. 2-Nitro-benzonitrile, **NRase I**, **NADH** and buffer were incubated. GCMS analysis (after 2 h) indicated the presence of *2-amino-benzamide* **284**.

Product conversion: 0.0 % (1 h), 4.5 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65 (M-CONH<sub>2</sub>-HCN).

2ii. 2-Nitro-benzonitrile, **NRase II**, **NADPH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *2-amino-benzamide* **284**.

Product conversion: 39.8 % (1 h), 75.4 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65 (M-CONH<sub>2</sub>-HCN).

2iii. 2-Nitro-benzonitrile, **NRase II**, **NADH** and buffer were incubated. GCMS analysis (after 2 h) indicated the presence of *2-amino-benzamide* **284**.

Product conversion: 0.0 % (1 h), 7.8 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65 (M-CONH<sub>2</sub>-HCN).

3ii. 2-Nitro-benzonitrile, **NRase III**, **NADPH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *2-amino-benzamide* **284**.

Product conversion: 31.4 % (1 h), 65.7 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65 (M-CONH<sub>2</sub>-HCN).

3iii. 2-Nitro-benzonitrile, **NRase III**, **NADH** and buffer were incubated. GCMS analysis (after 2 h) indicated the presence of *2-amino-benzamide* **283**.

Product conversion: 0.0 % (1 h), 6.5 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65 (M-CONH<sub>2</sub>-HCN).

4ii. 2-Nitro-benzonitrile, **glutathione reductase**, **NADPH** and buffer were incubated. GCMS analysis indicated the presence of the *starting material* **282**.

4iii. 2-Nitro-benzonitrile, **glutathione reductase**, **NADH** and buffer were incubated. GCMS analysis indicated the presence of the *starting material* **282**.



5ii. 2-Nitro-benzonitrile, **L-lactate dehydrogenase**, **NADPH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *2-amino-benzamide* **284**.

Product conversion: 28.2 % (1 h), 66.1 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65 (M-CONH<sub>2</sub>-HCN).

5iii. 2-Nitro-benzonitrile, **L-lactate dehydrogenase**, **NADH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *2-amino-benzamide* **284**.

Product conversion: 38.2 % (1 h), 84.8 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65 (M-CONH<sub>2</sub>-HCN).

6ii. 2-Nitro-benzonitrile, **P-450 Cytochrome reductase**, **NADPH** and buffer were incubated. GCMS analysis (after 1 h and 2 h) indicated the presence of *2-amino-benzamide* **283**.

Product conversion: 27.9 % (1 h), 26.4 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65 (M-CONH<sub>2</sub>-HCN).

6iii. 2-Nitro-benzonitrile, **P-450 Cytochrome reductase**, **NADH** and buffer were incubated. GCMS analysis (after 2 h) indicated the presence of *2-amino-benzamide* **283**.

Product conversion: 2.1 % (2 h);  $R_t$  15.0 min, oven temp 125 °C;  $m/z$  (EI) 136 ( $M^+$ ), 92 (M-CONH<sub>2</sub>), 65(M-CONH<sub>2</sub>-HCN).



## 7.7 General Procedure IV : Intermediate isolation.

A solution of the substrate (250.0 mg) in the minimum amount of dioxane was added to a suspension of *S. cerevisiae* (25.00 g) in water (100 mL) and incubated. The reduction of the substrate was monitored by HPLC (acetonitrile:water) or GCMS of 1 mL samples withdrawn at regular time intervals from the reaction mixture.

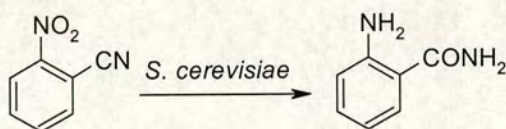
The reaction was stopped by the addition of ethyl acetate (100 mL) and the organic layer separated. The suspension was extracted with more ethyl acetate ( $3 \times 100$  mL) and the combined organic extracts were filtered through a bed of Celite<sup>TM</sup>, dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure to afford the crude product(s) as yellow/orange solid(s).

Purification of the crude was attempted by flash-column chromatography, preparative HPLC and LCMS techniques.

### 7.7.1 Label studies.

#### 7.7.1.1 Nitro-benzonitrile 282 studies.

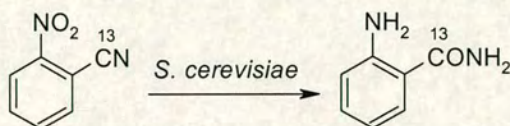
##### 2-Nitro-benzonitrile 282.



According to General Procedure VI, 2-nitro-benzonitrile **282** (88.8 mg, 0.6 mmol), *S. cerevisiae* (2.50 g) and water (10 mL) were incubated. After 2 h, HPLC indicated the presence of an unknown intermediate and 2-amino-benzamide. After work-up and purification afforded 2-amino-benzamide **284** as a pale yellow solid.

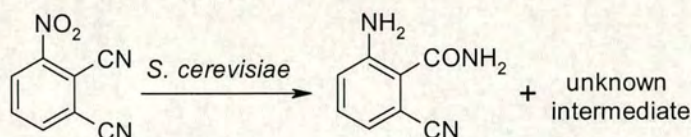
R<sub>f</sub> (EtOAc:Petrol, 1:1) 0.26; Mp 110.2-111.9 °C; Data as in **284**.



**<sup>13</sup>C-2-Nitro-benzonitrile 342.**

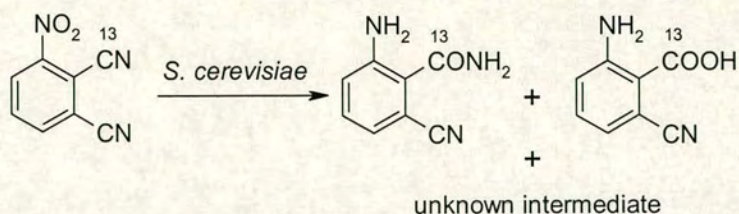
According to General Procedure VI, <sup>13</sup>C-2-nitro-benzonitrile **342** (44.7 mg, 0.3 mmol), *S. cerevisiae* (1.3 g) and water (5 mL) were incubated. The biotransformation was attempted several times, each time the reaction was stopped at different time intervals and work-up and purification afforded labelled 2-amino-benzamide **284** as a pale yellow solid.

*R<sub>f</sub>* (EtOAc:Petrol, 1:1) 0.27; Mp 110.5-112.2 °C; *m/z* (EI) 137 (*M*<sup>+</sup>); δ<sub>C</sub> ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 113.9 (<sup>13</sup>C), enhanced signal; Data as in **284**.

**7.7.1.2 Nitro-phthalonitrile 238 studies.****3-Nitro-phthalonitrile 238.**

According to General Procedure VI, 3-nitro-phthalonitrile **238** (100.0 mg, 0.6 mmol), *S. cerevisiae* (10.7 g) and water (42.9 mL) were incubated. After 30 min, HPLC indicated the presence of an unknown intermediate and 2-amino-6-cyano-benzamide **289**. After work-up and purification afforded a mixture of 2-amino-6-cyano-benzamide **289** and the unknown intermediate.



**2-<sup>13</sup>C-3-Nitro-phthalonitrile 343.**

According to General Procedure VI, 2-<sup>13</sup>C-3-nitro-phthalonitrile **343** (52.2 mg, 0.3 mmol), *S. cerevisiae* (5.4 g) and water (21.4 mL) were incubated. The biotransformation was attempted several times, each time the reaction was stopped at different time intervals and work-up and purification afforded 2-amino-6-cyano-benzamide **346**, 2-amino-6-cyano-benzoic acid **347** and the *unknown intermediate*.  
 $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 162.1, 171.2, 167.9 (<sup>13</sup>C), enhanced signals.



**7.8 General Procedure V : *Rhodococcus* sp. catalysed hydrolysis of (di)cyano-nitroarenes.<sup>225</sup>**

*Rhodococcus* sp. (0.5 mg of cells per mL of buffer) was suspended in phosphate buffer (0.1M, pH 7) and incubated in an orbital shaker at 30 °C and 200 rpm. The substrate (10.0 mmol) was added and the biotransformation monitored by HPLC/GCMS.

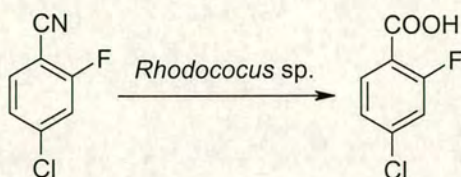
Upon complete substrate conversion, the buffer mixture was filtered through a Celite<sup>TM</sup> pad to remove the cells. The filtrate was basified using NaOH (pH 10, 2M) and extracted with ethyl acetate (3 x 30 mL). The combined organic extracts were washed with brine (50 mL), dried (MgSO<sub>4</sub>), filtered and concentrated under reduced pressure to afford any unreacted starting material.

The aqueous layer was acidified using HCl (pH 2, 2M) and extracted with ethyl acetate (3 x 30 mL). The combined organic extracts were washed with brine (50 mL), dried using MgSO<sub>4</sub>, filtered and concentrated under reduced pressure to afford the acid product(s). If necessary, further purification was achieved by preparative HPLC.

Quantities of substrate and buffer used, as well as reaction times, purification methods and yields are given for each individual biotransformation.

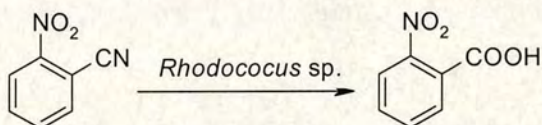


## 7.8.1 Hydrolysis of cyanonitroarenes.

4-Chloro-2-fluoro-benzoic acid **363**.

According to General Procedure V, 4-chloro-2-fluoro-benzonitrile **362** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (25.0 mg) and buffer (50 mL) were incubated for 90 min. Work-up afforded 4-chloro-2-fluoro-benzoic acid **363** as a pale brown solid (84.6 mg, 74.8 %).

Mp 134.2-135.8 °C, Lit.<sup>226</sup> 136.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3327, 3116 (br, O-H), 2692 (CH), 1682 (C=O), 1596 (Ph), 1257 (C-F);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 13.4 (1H, br s, COOH), 8.03 (1H, d, *J* 1.9, PhH), 7.92 (1H, d, *J* 8.8, PhH), 7.53 (1H, dd, *J* 8.3 and 1.9, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 165.5, 160.1 (C), 131.8, 130.6 (CH), 128.8, 119.5 (C), 117.3 (CH); *m/z* (EI) 174 (M<sup>+</sup>), 129 (M-CO<sub>2</sub>H), 109 (M-CO<sub>2</sub>H-HF); GC: *R*<sub>t</sub> 7.8 min, oven temp 150 °C.

2-Nitro-benzoic acid **364**.

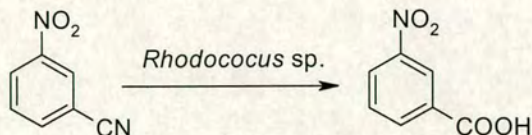
According to General Procedure V, 2-nitro-benzonitrile **282** (100.0 mg, 0.7 mmol), *Rhodococcus* sp. (25.0 mg) and buffer (50 mL) were incubated for 24 h. Work-up afforded 2-nitro-benzoic acid **364** as a pale yellow solid (37.2 mg, 32.8 %).

Mp 146.8-147.9 °C, Lit.<sup>227</sup> 147.0-148.5 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3200 (br, O-H), 2989 (CH), 1719 (C=O), 1589 (Ph), 1555, 1334 (NO<sub>2</sub>);  $\delta_{\text{H}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.60 (1H, br s, COOH), 7.90 (2H, m, PhH), 7.40 (2H, m, PhH);  $\delta_{\text{C}}$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 165.8, 148.3 (C), 132.3, 131.4, 129.6 (CH), 127.2 (C), 123.1 (CH); *m/z* (EI) 167



(M<sup>+</sup>), 149 (M-H<sub>2</sub>O), 119 (M-H<sub>2</sub>O-NO), 103 (M-H<sub>2</sub>O-NO<sub>2</sub>); GC: R<sub>t</sub> 10.8 min, oven temp 120 °C.

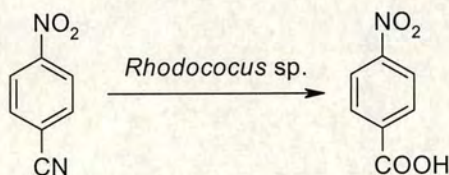
### 3-Nitro-benzoic acid **365**.



According to General Procedure V, 3-nitro-benzonitrile **285** (100.0 mg, 0.7 mmol), *Rhodococcus* sp. (25.0 mg) and buffer (50 mL) were incubated for 24 h. Work-up afforded 3-nitro-benzoic acid **365** as a white solid (61.6 mg, 53.7 %).

Mp 142.0 °C, Lit.<sup>228</sup> 142.0-144.0 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3259 (br, O-H), 3022 (CH), 1710 (C=O), 1592 (Ph), 1545, 1340 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{SO}, 200 \text{ MHz})$  14.0 (1H, br s, COOH), 8.58 (1H, dd, *J* 2.4 and 1.1, PhH), 8.44 (1H, td, *J* 7.8, 2.4 and 1.1, PhH), 8.32 (1H, td, *J* 7.8, 2.4 and 1.1, PhH), 7.79 (1H, dd, *J* 7.8, Ph);  $\delta_{\text{C}}((\text{CD}_3)_2\text{SO}, 63 \text{ MHz})$  165.3, 147.4 (C), 135.2 (CH), 131.9 (C), 130.0 (CH), 126.9, 123.3 (CH); *m/z* (EI) 167 (M<sup>+</sup>), 149 (M-H<sub>2</sub>O), 119 (M-H<sub>2</sub>O-NO), 103 (M-H<sub>2</sub>O-NO<sub>2</sub>); GC: R<sub>t</sub> 10.8 min, oven temp 120 °C.

### 4-Nitro-benzoic acid **366**.



According to General Procedure V, 4-nitro-benzonitrile **287** (100.0 mg, 0.7 mmol), *Rhodococcus* sp. (25.0 mg) and buffer (50 mL) were incubated for 24 h. Work-up afforded 4-nitro-benzoic acid **366** as a white solid (58.4 mg, 51.4 %).

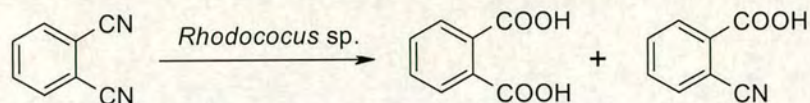
Mp 240.0-241.5 °C, Lit.<sup>229</sup> 241.0-243.0 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3185 (br, O-H), 2932 (CH), 1730 (C=O), 1601 (Ph), 1530, 1332 (NO<sub>2</sub>);  $\delta_{\text{H}}((\text{CD}_3)_2\text{SO}, 200 \text{ MHz})$  11.2 (1H, s, COOH), 8.33 (2H, d, *J* 8.9, PhH), 8.31 (2H, d, *J* 8.9, PhH);  $\delta_{\text{C}}((\text{CD}_3)_2\text{SO}, 63$



MHz) 165.2, 150.1, 136.5 (C), 130.2, 123.0 (CH);  $m/z$  (EI) 167 ( $M^+$ ), 149 ( $M-H_2O$ ), 119 ( $M-H_2O-NO$ ), 103 ( $M-H_2O-NO_2$ ); GC:  $R_t$  10.8 min, oven temp 120 °C.

### 7.8.2 Hydrolysis of dicyanonitroarenes.

#### Phthalic acid **375** and 2-Cyano-benzoic acid **376**.

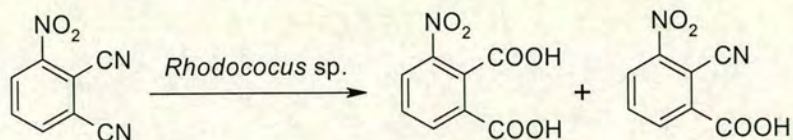


According to General Procedure V, 1,2-dicyano-benzene **335** (100.3 mg, 0.8 mmol), *Rhodococcus* sp. (35.0 mg) and buffer (70 mL) were incubated for 24 h. After work-up, purification by preparative HPLC (acetonitrile:water, 20:80) afforded *phthalic acid* **375** as a white solid (35.6 mg, 26.8 %) and *2-cyano-benzoic acid* **376** as a pale yellow solid (57.5 mg, 48.9 %).

Phthalic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 3.1 min; Mp 210.2 °C, Lit.<sup>230</sup> 211.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3302, 3258 (br, O-H), 3026 (CH), 1674 (C=O), 1622 (Ph);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.20 (2H, br s, COOH), 7.65 (2H, d,  $J$  9.1, PhH), 7.58 (2H, d,  $J$  9.1, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 167.8, 131.7 (C), 129.8, 127.6 (CH);  $m/z$  (ES<sup>-</sup>) 165 ( $M-H$ )<sup>-</sup>, 148 ( $M-H_2O$ ), 130 ( $M-2H_2O$ ).

2-Cyano-benzoic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 7.8 min; Mp 183.1-184.7 °C, Lit.<sup>231</sup> 183.0-185.0 °C;  $\nu_{\max}(\text{KBr})/\text{cm}^{-1}$  3302, 3258 (br, O-H), 3026 (CH), 1674 (C=O), 1622 (Ph);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.02 (2H, m, PhH), 7.83 (2H, m, PhH), 7.70 (1H, br s, COOH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 165.4, 135.8 (C), 135.1, 133.7, 132.9, 130.8 (CH), 119.4, 113.5 (C);  $m/z$  (ES<sup>-</sup>) 147 ( $M-H$ )<sup>-</sup>, 129 ( $M-H_2O$ ), 99 ( $M-NO$ ).

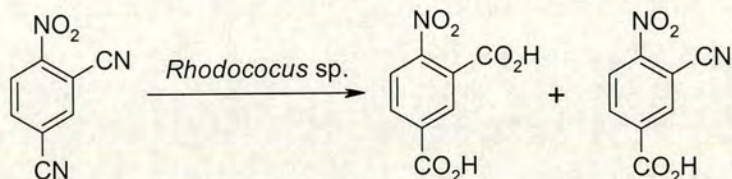


**3-Nitro-phthalic acid 234 and 2-Cyano-3-nitro-benzoic acid 377.**

According to General Procedure V, 3-nitro-phthalonitrile **238** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg, 1.5 eq.) and buffer (250 mL) were incubated for 7 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 3-nitro-phthalic acid **234** as a yellow solid (14.0 mg, 11.0 %) and 2-cyano-3-nitro-benzoic acid **377** as a yellow solid (24.5 mg, 22.0 %).

3-Nitro-phthalic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 3.6 min; Mp 219.2-221.8 °C, Lit.<sup>232</sup> 220.0-222.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3320, 3177 (br, O-H), 3101 (CH), 1766, 1735 (C=O), 1610 (Ph), 1552, 1350 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.89 (2H, br s, COOH), 8.32 (1H, dd,  $J$  8.2 and 1.3, PhH), 8.23 (1H, dd,  $J$  8.0 and 1.3, PhH), 7.80 (1H, dd,  $J$  8.2 and 8.0, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 170.3, 145.9 (C), 134.3, 132.2 (CH), 130.8 (C), 129.8 (CH), 128.7 (C);  $m/z$  (ES<sup>-</sup>) 210 (M-H)<sup>-</sup>, 165 (M-CO<sub>2</sub>).

2-Cyano-3-nitro-benzoic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 0.5 min; Mp 184-186 °C;  $\nu_{\max}$  (KBr)/cm<sup>-1</sup> 3268 (br, O-H), 3028 (CH), 2296 (CN), 1699 (C=O), 1625 (Ph), 1542, 1349 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.11 (1H, br s, COOH), 8.30 (1H, dd,  $J$  8.0 and 1.5, PhH), 8.21 (1H, dd,  $J$  7.8 and 1.5, PhH), 7.74 (1H, dd,  $J$  8.0 and 7.8, PhH);  $\delta_C$ ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 170.9, 155.2 (C), 138.5 (CH), 131.1 (C), 130.9, 128.2 (CH), 120.9, 113.7 (C);  $m/z$  (ES<sup>-</sup>) 191 (M-H)<sup>-</sup>, 146 (M-NO<sub>2</sub>), 119 (M-NO<sub>2</sub>-NH<sub>2</sub>), Found (M-H)<sup>-</sup> 191.0891, C<sub>8</sub>H<sub>4</sub>N<sub>2</sub>O<sub>4</sub> requires 191.0093.

**4-Nitro-isophthalic acid 232 and 3-Cyano-4-nitro-benzoic acid 378.**

According to General Procedure V, 4-nitro-isophthalonitrile **233** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg) and buffer (250 mL) were incubated for 24

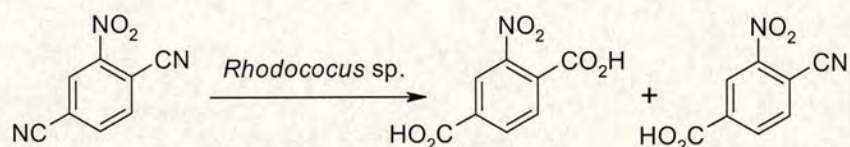


h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded *4-nitro-isophthalic acid* **232** as a yellow solid (18.8 mg, 15.4 %) and *3-cyano-4-nitro-benzoic acid* **378** as a pale yellow solid (74.5 mg, 67.1 %).

4-Nitro-isophthalic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 4.0 min; Mp 245.2-247.8 °C, Lit.<sup>172</sup> 245.0-246.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3350 (br, O-H), 3087 (CH), 1718, 1685 (C=O), 1617 (Ph), 1522, 1354 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.88 (1H, d,  $J$  1.7, PhH), 8.22 (1H, dd,  $J$  8.2 and 1.7, PhH), 8.02 (1H, d,  $J$  1.7, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 165.8, 165.2, 151.0, 134.2 (C), 130.9, 128.9 (CH), 126.9 (C), 124.2 (CH);  $m/z$  (ES<sup>-</sup>) 210 (M-H)<sup>-</sup>, 165 (M-CO<sub>2</sub>).

3-Cyano-4-nitro-benzoic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 5.4 min; Mp 198.3-199.9 °C, (Found C, 48.41; H, 14.82; N, 33.00; C<sub>8</sub>H<sub>4</sub>N<sub>2</sub>O<sub>4</sub> requires C, 50.21; H, 14.59; N, 33.32);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3306, 3179 (br, O-H), 3073 (CH), 2245 (CN), 1700, 1684 (C=O), 1603 (Ph), 1533, 1351 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.62 (1H, br s, COOH), 8.52 (1H, d,  $J$  1.8, PhH), 8.35 (1H, d,  $J$  8.6, PhH), 8.48 (1H, dd,  $J$  8.6, and 1.8, PhH);  $\delta_C$ ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 164.6, 149.5, 139.5 (C), 134.5, 133.5, 126.2 (CH), 115.4, 107.3 (C);  $m/z$  (ES<sup>-</sup>) 191 (M-H)<sup>-</sup>, 146 (M-NO<sub>2</sub>), Found (M-H)<sup>-</sup> 191.1824, C<sub>8</sub>H<sub>4</sub>N<sub>2</sub>O<sub>4</sub> requires 191.0093.

## 2-Nitro-terephthalic acid **222** and 4-Cyano-3-nitro-benzoic acid **379**.



According to General Procedure V, 2-nitro-terephthalonitrile **225** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg) and buffer (250 mL) were incubated for 10 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded *2-nitro-terephthalic acid* **222** as a yellow solid (18.8 mg, 15.4 %) and *4-cyano-3-nitro-benzoic acid* **379** as a pale yellow solid (78.1 mg, 70.4 %).

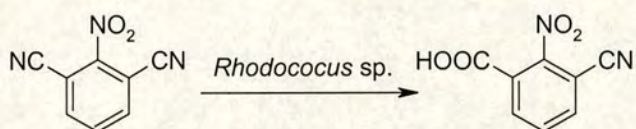
2-Nitro-terephthalic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 4.0 min; Mp 264.5-266.1 °C, Lit.<sup>233</sup> 266.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3359, 3196 (br, O-H), 3109 (CH), 1700, 1662 (C=O), 1616 (Ph), 1533, 1350 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.05 (1H, d,  $J$  1.5,



PhH), 8.50 (1H, dd,  $J$  7.8 and 1.5, PhH), 8.25 (1H, d,  $J$  7.9, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 166.8, 165.3, 147.1, 136.2 (C), 132.2 (CH), 131.9 (C), 129.3, 123.0 (CH);  $m/z$  (ES<sup>-</sup>) 210 (M-H)<sup>-</sup>, 165 (M-CO<sub>2</sub>).

4-Cyano-3-nitro-benzoic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 5.4 min; Mp 204.0-205.6 °C, Lit.<sup>234</sup> 205.0-206.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3376, 3184 (br, O-H), 2238 (CN), 1675 (C=O), 1625 (Ph), 1539, 1343 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 10.30 (1H, br s, COOH), 9.13 (1H, d,  $J$  1.5, PhH), 8.39 (1H, dd,  $J$  8.0 and 1.5, PhH), 8.16 (1H, d,  $J$  8.0, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 164.5, 148.6 (C), 136.3 (CH), 135.9 (C), 133.2, 124.2 (CH), 115.4, 109.2 (C);  $m/z$  (ES<sup>-</sup>) 191 (M-H)<sup>-</sup>, 174 (M-H<sub>2</sub>O), 128 (M-H<sub>2</sub>O-NO<sub>2</sub>).

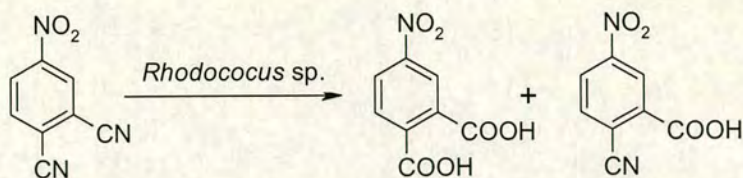
### 3-Cyano-2-nitro-benzoic acid 386.



According to General Procedure V, 2-nitro-isophthalonitrile **230** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg) and buffer (250 mL) were incubated for 5 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 3-cyano-2-nitro-benzoic acid **386** as a white solid (80.0 mg, 72.1 %).

3-Cyano-2-nitro-benzoic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 4.5 min; Mp 184.0-186.0 °C, (Found C, 49.92; H, 12.69; N, 33.57; C<sub>8</sub>H<sub>4</sub>N<sub>2</sub>O<sub>4</sub> requires C, 50.21; H, 14.59; N, 33.32);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3309 (br, O-H), 3193 (CH), 2241 (CN), 1673 (C=O), 1605 (Ph), 1536, 1355 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.42 (1H, br s, COOH), 8.39 (1H, dd,  $J$  7.7 and 1.0, PhH), 8.06 (1H, dd,  $J$  8.0 and 1.0, PhH), 7.93 (1H, dd,  $J$  8.0 and 7.7, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 165.0, 149.3 (C), 136.0, 133.9, 133.0 (CH), 130.2, 114.1, 106.4 (C);  $m/z$  (ES<sup>-</sup>) 191 (M-H)<sup>-</sup>, 146 (M-NO<sub>2</sub>), Found (M-H)<sup>-</sup> 190.9892, C<sub>8</sub>H<sub>4</sub>N<sub>2</sub>O<sub>4</sub> requires 191.0093.

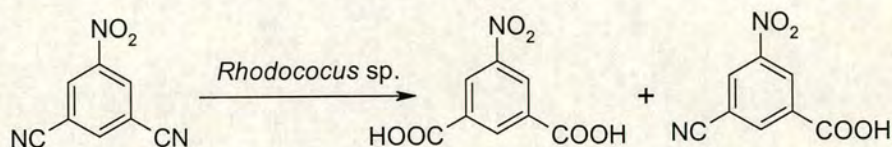


**4-Nitro-phthalic acid 388 and 2-Cyano-5-nitro-benzoic acid 377.**

According to General Procedure V, 4-nitro-phthalonitrile **294** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg) and buffer (250 mL) were incubated for 12 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 4-nitro-phthalic acid **388** as a pale orange solid (6.0 mg, 8.0 %) and 6-cyano-3-nitro-benzoic acid **387** as a yellow solid (65.0 mg, 59.0 %).

4-Nitro-phthalic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 4.8 min; Mp 163.0-164.5 °C, Lit.<sup>235</sup> 163.0-165.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3330 (br, O-H), 3112 (CH), 1792, 1700 (C=O), 1622 (Ph), 1547, 1350 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.91 (1H, br s, COOH), 9.05 (1H, d,  $J$  2.3, PhH), 9.00 (1H, br s, COOH), 8.68 (1H, dd,  $J$  8.6 and 2.3, PhH), 8.43 (1H, d,  $J$  8.6, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 167.5, 147.5, 138.9, 132.9 (C), 129.2, 125.3, 123.1 (CH);  $m/z$  (ES<sup>-</sup>) 210 (M-H)<sup>-</sup>, 165 (M-CO<sub>2</sub>).

6-Cyano-3-nitro-benzoic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 7.6 min; Mp 212.0-214.0 °C;  $\nu_{\max}$  (KBr)/cm<sup>-1</sup> 3259 (br, O-H), 2987 (CH), 2314 (CN), 1687 (C=O), 1610 (Ph), 1561, 1346 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.91 (1H, d,  $J$  2.0, PhH), 8.69 (1H, br s, COOH), 8.57 (1H, dd,  $J$  8.0 and 2.0, PhH), 8.15 (1H, d,  $J$  8.0, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 164.8, 145.2, 135.4 (C), 134.2, 128.9, 126.3 (CH), 124.6, 118.7 (C);  $m/z$  (ES<sup>-</sup>) 191 (M-H)<sup>-</sup>, 146 (M-NO<sub>2</sub>), Found (M-H)<sup>-</sup> 191.0177, C<sub>8</sub>H<sub>4</sub>N<sub>2</sub>O<sub>4</sub> requires 191.0093.

**5-Nitro-isophthalic acid 218 and 5-Cyano-3-nitro-benzoic acid 389.**

According to General Procedure III, 5-nitro-isophthalonitrile **221** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg) and buffer (250 mL) were incubated for



12 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 5-nitro-isophthalic acid **218** as a pale yellow solid (20.0 mg, 15.0 %) and 5-cyano-3-nitro-benzoic acid **389** as a yellow solid (77.0 mg, 69.0 %).

5-Nitro-isophthalic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 3.4 min; Mp 255.9-256.8 °C, Lit.<sup>163</sup> 256.0-258.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3456, 3347 (br, O-H), 3087 (CH), 1717, 1685 (C=O), 1616 (Ph), 1522, 1355 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 10.2 (2H, br s, COOH), 8.81 (2H, d,  $J$  1.4, PhH), 8.79 (1H, t,  $J$  1.4, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 165.5, 148.0, 136.2 (C), 132.8, 124.6 (CH);  $m/z$  (ES<sup>-</sup>) 210 (M-H)<sup>-</sup>, 165 (M-CO<sub>2</sub>).

5-Cyano-3-nitro-benzoic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 40:60) 7.0 min; Mp 170.0 °C, Lit.<sup>163</sup> 170.0-171.0 °C;  $\nu_{\max}$ (KBr, cm<sup>-1</sup>) 3395 (br, O-H), 3079 (CH), 2250 (CN), 1672 (C=O), 1617 (Ph), 1540, 1353 (NO<sub>2</sub>);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.85 (1H, br s, COOH), 9.12 (1H, dd,  $J$  1.5, PhH), 8.89 (1H, dd,  $J$  1.5, PhH), 8.69 (1H, dd,  $J$  1.5, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 168.2, 148.3 (C), 137.0, 135.1 (CH), 135.0 (C), 129.9 (CH), 116.8, 113.2 (C);  $m/z$  (EI) 192 (M<sup>+</sup>), 146 (M-NO<sub>2</sub>).



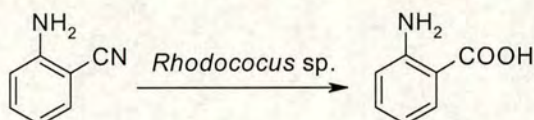
## 7.9 General Procedure VI : *Rhodococcus* sp. catalysed hydrolysis of *S. cerevisiae* products.

*Rhodococcus* sp. (0.5 mg of cells per mL of buffer) was suspended in phosphate buffer (0.1M, pH 7) and incubated in an orbital shaker at 30 °C and 200 rpm. The substrate was added and the biotransformation monitored by HPLC. Upon complete substrate conversion, the reaction was filtered through a Celite<sup>TM</sup> pad to remove the enzyme. The aqueous filtrate was concentrated under reduced pressure to yield the crude amino acid derivative(s) which were purified by preparative HPLC.

Quantities of substrate and buffer used, as well as reaction times, purification conditions and yields are given for each individual biotransformation.

### 7.9.1 Hydrolysis of amino-benzonitriles.

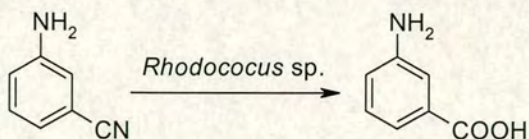
#### 2-Amino-benzoic acid **367**.



According to General Procedure VI, 2-amino-benzonitrile **283** (100.0 mg, 0.9 mmol), *Rhodococcus* sp. (25.0 mg) and buffer (50 mL) were incubated for 24 h. After work-up, purification by preparative HPLC (acetonitrile:water, 20:80) afforded 2-amino-benzoic acid **367** as a cream solid (40.7 mg, 35.0 %).

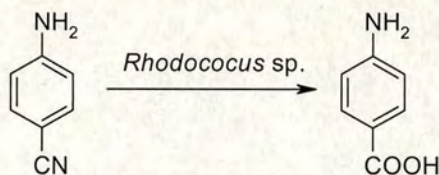
$R_t$  (MeCN:H<sub>2</sub>O, 40:60) 4.8 min; Mp 146.5 °C, Lit.<sup>236</sup> 145.0-148.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3624, 3542 (NH<sub>2</sub>), 3203 (br, O-H), 3022 (CH), 1705 (C=O), 1612 (Ph), 1525, 1355 (NO<sub>2</sub>);  $\delta_H$  (CDCl<sub>3</sub>, 200 MHz) 8.60 (1H, br s, COOH), 7.88 (1H, dd,  $J$  8.4, 8.2 and 1.9, PhH), 7.25 (1H, td,  $J$  8.4, 8.2 and 1.9, PhH), 7.65 (2H, m, PhH), 7.25 (1H, td,  $J$  8.4 and 1.9, PhH);  $\delta_C$  (CDCl<sub>3</sub>, 63 MHz) 168.9, 151.2 (C), 132.9, 131.2, 115.8, 114.1 (CH), 108.3 (C);  $m/z$  (EI) 137 (M<sup>+</sup>), 120 (M-NH<sub>3</sub>), 102 (M-NH<sub>3</sub>-H<sub>2</sub>O).



**3-Amino-benzoic acid 368.**

According to General Procedure VI, 3-amino-benzonitrile **286** (100.0 mg, 0.9 mmol), *Rhodococcus* sp. (25.0 mg) and buffer (50 mL) were incubated for 24 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 3-amino-benzoic acid **368** as a white solid (83.8 mg, 72.0 %).

$R_t$  (MeCN:H<sub>2</sub>O, 40:60) 4.2 min; Mp 175.5 °C, Lit.<sup>237</sup> 175.0-177.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3144 (br, O-H), 1682 (C=O), 1608 (Ph), 1531, 1342 (NO<sub>2</sub>);  $\delta_H$  (CDCl<sub>3</sub>, 200 MHz) 7.18 (1H, dd,  $J$  1.4 and 1.2, PhH), 7.09 (2H, m,  $J$  8.7, PhH), 6.78 (1H, td,  $J$  6.8, 6.1 and 1.2, PhH);  $\delta_C$  (CDCl<sub>3</sub>, 63 MHz) 169.8, 147.9, 131.0 (C), 128.2, 118.0, 117.1, 114.4 (CH);  $m/z$  (EI) 137 (M<sup>+</sup>), 120 (M-NH<sub>3</sub>), 102 (M-NH<sub>3</sub>-H<sub>2</sub>O).

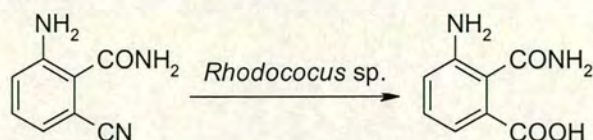
**4-Amino-benzoic acid 369.**

According to General Procedure VI, 4-amino-benzonitrile **288** (100.0 mg, 0.9 mmol), *Rhodococcus* sp. (25.0 mg) and buffer (50 mL) were incubated for 24 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 4-amino-benzoic acid **369** as a yellow solid (64.3 mg, 55.2 %).

$R_t$  (MeCN:H<sub>2</sub>O, 40:60) 3.6 min; Mp 186.2-187.5 °C, Lit.<sup>238</sup> 187.0-190.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3685, 3508 (NH<sub>2</sub>), 3152 (br, O-H), 1692 (C=O), 1622 (Ph), 1520, 1342 (NO<sub>2</sub>);  $\delta_H$  (CDCl<sub>3</sub>, 200 MHz) 7.65 (2H, d,  $J$  8.0, PhH), 6.59 (2H, d,  $J$  8.0, PhH), 4.10 (2H, br s, NH<sub>2</sub>);  $\delta_C$  (CDCl<sub>3</sub>, 63 MHz, CDCl<sub>3</sub>) 167.3 (C), 152.2, 131.2 (CH), 117.0 (C), 112.0 (CH);  $m/z$  (EI) 137 (M<sup>+</sup>), 120 (M-NH<sub>3</sub>), 102 (M-NH<sub>3</sub>-H<sub>2</sub>O).

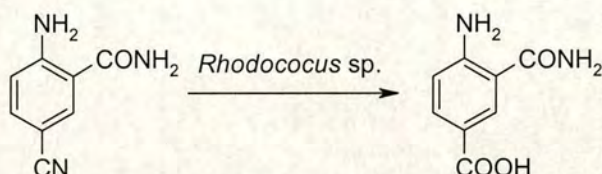


## 7.9.2 Hydrolysis of amino/(amido)-benzonitriles.

3-Amino-phthalamic acid **381**.

According to General Procedure VI, 2-amino-6-cyano-benzamide **289** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg, 1.3 eq.) and buffer (250mL) were incubated for 48 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 3-amino-phthalamic acid **381** as a yellow solid (31.8 mg, 29.4 %).

$R_t$  (MeCN:H<sub>2</sub>O, 20:80) 6.1 min; Mp 172.0-174.5 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3421, 3258 (NH<sub>2</sub>), 3246 (br, O-H), 1698 (C=O), 1664 (NH), 1619 (Ph);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.89 (1H, dd,  $J$  6.9 and 0.8, PhH), 7.55 (1H, dd,  $J$  6.9 and 6.7, PhH), 7.02 (1H, dd,  $J$  6.7 and 0.8, PhH);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 167.8, 166.5, 143.8 (C), 136.4 (CH), 130.5, 123.8 (C), 125.8, 123.2 (CH);  $m/z$  (EI) 180 (M<sup>+</sup>), 162 (M-H<sub>2</sub>O), 144 (M-2H<sub>2</sub>O), 96 (M-2H<sub>2</sub>O-NO), Found (M<sup>+</sup>) 180.0498 C<sub>8</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub> requires 180.0535.

4-Amino-isophthalamic acid **382**.

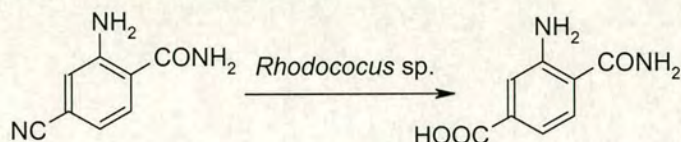
According to General Procedure VI, 2-amino-5-cyano-benzamide **291** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg) and buffer (250mL) were incubated for 24 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 4-amino-isophthalamic acid **382** as an orange solid (78.9 mg, 66.9 %).

$R_t$  (MeCN:H<sub>2</sub>O, 40:60) 3.6 min; Mp 274.0-275.3 °C; (Found C, 50.92; H, 4.27; N, 17.45; C<sub>8</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub> requires C, 53.32; H, 4.48; N, 15.55);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3515, 3377 (br, O-H, NH<sub>2</sub>), 3190 (CH), 1682 (C=O, NH), 1651 (Ph);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.89 (1H, br s, COOH), 7.80 (1H, d,  $J$  1.5, PhH), 7.31 (1H, br s, CONH<sub>2</sub>), 7.16 (1H,



dd,  $J$  8.2 and 1.5, PhH), 6.93 (1H, d,  $J$  8.2, PhH), 6.66 (1H, br s, CONH<sub>2</sub>), 3.5 (2H, br s, NH<sub>2</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 170.9, 168.1, 150.1 (C), 137.5, 128.8 (CH), 118.2, 115.7 (C), 113.1 (CH);  $m/z$  (EI) 205 (MNa<sup>+</sup>), 163 (M-H<sub>2</sub>O), Found (M<sup>+</sup>) 180.04341 C<sub>8</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub> requires 180.0535.

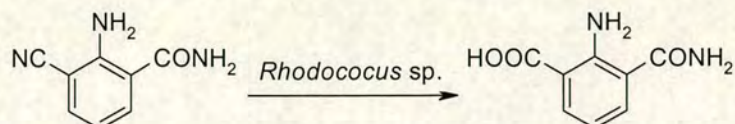
### 3-Amino-terephthalamic acid 383.



According to General Procedure VI, using 2-amino-4-cyano-benzamide **292** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg) and buffer (250mL) were incubated for 24 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 3-amino-terephthalamic acid **383** as a pale orange solid (60.3 mg, 69.7 %).

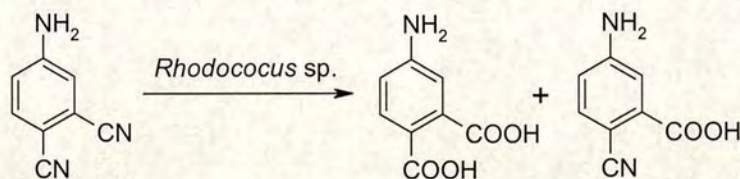
$R_t$  (MeCN:H<sub>2</sub>O, 40:60) 6.0 min; Mp 280.0-282.0 °C, (Found C, 53.11; H, 4.33; N, 15.81; C<sub>8</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub> requires C, 53.32; H, 4.48; N, 15.55);  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3498, 3379 (br, O-H, NH<sub>2</sub>), 3028 (CH), 1652 (C=O, NH), 1589 (Ph);  $\delta_H$  ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 7.92 (1H, br s, COOH), 7.62 (1H, d,  $J$  8.2, PhH), 7.36 (1H, br s, CONH<sub>2</sub>), 7.26 (1H, dd,  $J$  8.2 and 1.5, PhH), 7.14 (1H, br s, CONH<sub>2</sub>), 7.05 (1H, d,  $J$  1.5, PhH), 3.40 (2H, br s, NH<sub>2</sub>);  $\delta_C$  ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 170.5, 167.8, 148.2, 137.5 (C), 128.8 (CH), 125.0 (C), 122.1, 114.4 (CH);  $m/z$  (EI) 205 (MNa<sup>+</sup>), 180 (M<sup>+</sup>), 163 (M-H<sub>2</sub>O), Found (M<sup>+</sup>) 180.0427, C<sub>8</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub> requires 180.0535.



**2-Amino-isophthalamic acid 391.**

According to General Procedure VI, using 2-amino-3-cyano-benzamide **293** (25.0 mg, 0.2 mmol), *Rhodococcus* sp. (38.0 mg) and buffer (75 mL) were incubated for 3 d. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 2-amino-isophthalamic acid **391** as a pale yellow gum (25.0 mg, 89.0 %).

$R_t$  (MeCN:H<sub>2</sub>O, 15:85) 3.4 min;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3422, 3251 (br, O-H, NH<sub>2</sub>), 2987 (CH), 1683 (C=O, NH), 1678 (Ph);  $\delta_H$ ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 8.97 (1H, br s, COOH), 7.97 (1H, dd,  $J$  7.7 and 1.1, PhH), 7.84 (1H, br s, CONH<sub>2</sub>), 7.66 (1H, dd,  $J$  7.6 and 1.1, PhH), 6.48 (1H, dd,  $J$  7.7 and 7.6, PhH), 5.25 (2H, br s, NH<sub>2</sub>);  $\delta_C$ ((CD<sub>3</sub>)<sub>2</sub>SO, 63 MHz) 171.9, 170.3, 151.6 (C), 133.2, 132.9 (CH), 119.8 (C), 116.6 (CH), 113.6 (C);  $m/z$  (EI) 205 (MNa<sup>+</sup>), Found (M<sup>+</sup>) 180.0490, C<sub>8</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub> requires 180.0535.

**4-Amino-phthalic acid 394 and 4-Amino-2-cyano-benzoic acid 392.**

According to General Procedure VI, 4-amino-isophthalonitrile **295** (100.0 mg, 0.6 mmol), *Rhodococcus* sp. (125.0 mg) and buffer (250 mL) were incubated for 48 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 4-amino-phthalic acid **394** as a pale yellow solid (15.7 mg, 14.3 %) and 3-amino-6-cyano-benzoic acid **392** as a pale orange solid (52.0 mg, 52.8 %).

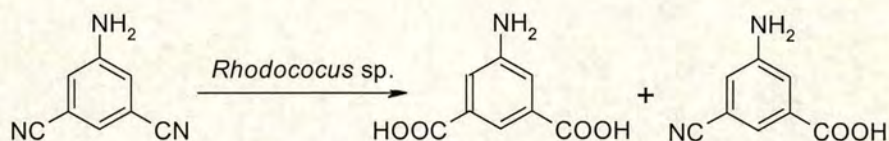
4-Amino-phthalic acid:  $R_t$  (MeCN:H<sub>2</sub>O, 20:80) 5.8 min; Mp > 300.0 °C, Lit.<sup>239</sup> > 300.0 °C;  $\nu_{\max}$ (KBr)/cm<sup>-1</sup> 3422, 3215 (NH<sub>2</sub>), 3208 (br, O-H), 2982 (CH), 1754 (C=O), 1619 (Ph);  $\delta_H$ ((CD<sub>3</sub>)<sub>2</sub>SO, 200 MHz) 9.23 (1H, br s, COOH), 8.22 (1H, d,  $J$  8.1, PhH), 8.13 (1H, d,  $J$  2.1, PhH), 8.02 (1H, dd,  $J$  8.1 and 2.1, PhH), 6.89 (2H, br s,



$\text{NH}_2$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 166.3, 158.2, 138.0 (C), 134.2 (CH), 125.4 (C), 123.5, 119.8 (CH);  $m/z$  ( $\text{FAB}^+$ ) 182 ( $\text{MH}^+$ ).

3-Amino-6-cyano-benzoic acid:  $R_t$  ( $\text{MeCN}:\text{H}_2\text{O}$ , 20:80) 8.6 min; Mp 162.0-163.0 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3456, 3375 ( $\text{NH}_2$ ), 2215 (CN), 1700 ( $\text{C}=\text{O}$ ), 1598 (Ph);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 8.10 (1H, br s,  $\text{COOH}$ ), 7.63 (1H, d,  $J$  2.3,  $\text{PhH}$ ), 7.02 (1H, d,  $J$  8.7,  $\text{PhH}$ ), 6.83 (1H, dd,  $J$  8.7 and 2.3,  $\text{PhH}$ ), 6.74 (2H, br s,  $\text{NH}_2$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 169.3, 153.3, 140.0 (C), 135.1, 125.7, 120.3 (CH), 116.6, 110.3 (C), 97.8 (C);  $m/z$  (EI) 162 ( $\text{M}^+$ ), 144 ( $\text{M}-\text{H}_2\text{O}$ ), Found ( $\text{M}^+$ ) 162.0434,  $\text{C}_8\text{H}_6\text{N}_2\text{O}_3$  requires 162.0430.

### 5-Amino-isophthalic acid **395** and 3-Amino-5-cyano-benzoic acid **393**.



According to General Procedure VI, 5-amino-isophthalonitrile **296** (100.0 mg, 0.8 mmol), *Rhodococcus* sp. (125.0 mg) and buffer (250 mL) were incubated for 3 d. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 5-amino-isophthalic acid **395** as a white solid (20.0 mg, 15.8 %) and 3-amino-5-cyano-benzoic acid **393** as a pale orange solid (55.0 mg, 48.7 %).

5-Amino-isophthalic acid:  $R_t$  ( $\text{MeCN}:\text{H}_2\text{O}$ , 10:90) 4.7 min; Mp > 300.0 °C, Lit.<sup>240</sup> > 300.0 °C;  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3204, 3346 ( $\text{NH}_2$ ), 2218 (CN), 1682 ( $\text{C}=\text{O}$ , NH), 1616 (Ph);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 9.52 (2H, br s,  $\text{COOH}$ ), 7.88 (2H, d,  $J$  1.3,  $\text{PhH}$ ), 8.16 (1H, s,  $J$  1.3,  $\text{PhH}$ ), 6.17 (2H, br s,  $\text{NH}_2$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 169.3, 150.4, 135.2 (C), 121.2, 120.3 (CH);  $m/z$  ( $\text{FAB}^+$ ) 182 ( $\text{MH}^+$ ).

3-Amino-5-cyano-benzoic acid:  $R_t$  ( $\text{MeCN}:\text{H}_2\text{O}$ , 10:90) 6.0 min; Mp 172.0-174.4 °C, (Found C, 59.13; H, 3.42; N, 17.53;  $\text{C}_8\text{H}_6\text{N}_2\text{O}_2$  requires C, 59.26; H, 3.73; N, 17.28);  $\nu_{\text{max}}(\text{KBr})/\text{cm}^{-1}$  3501, 3346 (br, O-H,  $\text{NH}_2$ ), 3129 (CH), 2230 (CN), 1687 ( $\text{C}=\text{O}$ ), 1598 (Ph);  $\delta_{\text{H}}$  ( $(\text{CD}_3)_2\text{SO}$ , 200 MHz) 7.56 (1H, dd,  $J$  1.8,  $\text{PhH}$ ), 7.44 (1H, dd,  $J$  1.8,  $\text{PhH}$ ), 7.02 (1H, dd,  $J$  1.8,  $\text{PhH}$ ) 6.01 (2H, br s,  $\text{NH}_2$ );  $\delta_{\text{C}}$  ( $(\text{CD}_3)_2\text{SO}$ , 63 MHz) 169.8,



150.0, 138.2 (C), 128.9, 126.2, 124.3 (CH), 114.9, 113.5 (C);  $m/z$  (FAB<sup>+</sup>) 205 (MNa<sup>+</sup>), Found (MH<sup>+</sup>) 163.0500, C<sub>8</sub>H<sub>6</sub>N<sub>2</sub>O<sub>2</sub> requires 163.0508.

### 7.10 General Procedure VII : *S. cerevisiae* catalysed reduction of *Rhodococcus* sp. products.

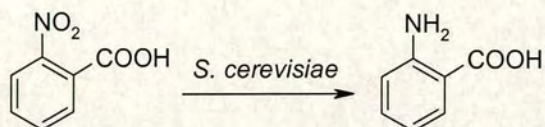
A solution of the substrate (250.0 mg) in the minimum amount of dioxane was added to a suspension of *S. cerevisiae* (25.00 g) in water (100 mL) and incubated. The reduction of the substrate was monitored by HPLC (acetonitrile:water) of 1 mL samples withdrawn at regular time intervals from the reaction mixture. Each sample was microcentrifuged, 400  $\mu$ L of the aqueous layer was removed and filtered before being diluted with 600  $\mu$ L of mobile phase and subjected to HPLC analysis.

Upon complete substrate consumption, the *S. cerevisiae* suspension was centrifuged and the aqueous layer was removed and filtered through a Celite<sup>TM</sup> pad. The filtrate collected was concentrated under reduced pressure to yield crude amino-acid derivatives, which were purified by preparative HPLC.

Quantities of substrate and *S. cerevisiae* used, as well as reaction times, purification conditions and yields are given for each individual biotransformation.

#### 7.10.1 Reduction of nitro-benzoic acids.

##### 2-Amino-benzoic acid 367.



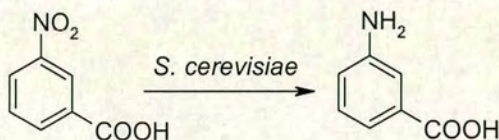
According to General Procedure VII, 2-nitro-benzoic acid **364** (100.0 mg, 0.6 mmol), *S. cerevisiae* (10.00 g) and water (40 mL) were incubated for 24 h. After work-up,



purification by preparative HPLC (acetonitrile:water, 20:80) afforded 2-amino-benzoic acid **367** as a cream solid (45.4 mg, 55.2 %).

$R_t$  (MeCN:H<sub>2</sub>O, 40:60) 4.8 min; Mp 146.5 °C; Data as in **367**.

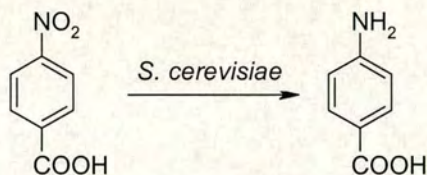
### 3-Amino-benzoic acid **368**.



According to General Procedure VII, 3-nitro-benzoic acid **365** (100.0 mg, 0.6 mmol), *S. cerevisiae* (10.00 g) and water (40 mL) were incubated for 24 h. After work-up, purification by preparative HPLC (acetonitrile:water, 20:80) afforded 3-amino-benzoic acid **368** as a white solid (41.6 mg, 50.6 %).

$R_t$  (MeCN:H<sub>2</sub>O, 40:60) 4.2 min; Mp 175.5 °C; Data as in **368**.

### 4-Amino-benzoic acid **369**.

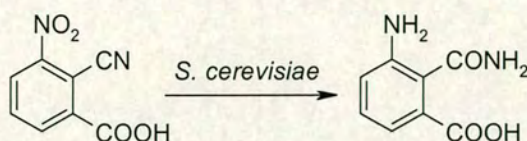


According to General Procedure VII, 4-nitro-benzoic acid **366** (100.0 mg, 0.6 mmol), *S. cerevisiae* (10.00 g) and water (40 mL) were incubated for 24 h. After work-up, purification by preparative HPLC (acetonitrile:water, 20:80) afforded 4-amino-benzoic acid **369** as a yellow solid (45.0 mg, 54.7 %).

$R_t$  (MeCN:H<sub>2</sub>O, 40:60) 3.6 min; Mp 186.2-187.5 °C; Data as in **369**.

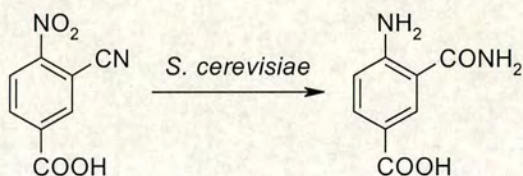


## 7.10.2 Reduction of nitro-cyano-benzoic acids.

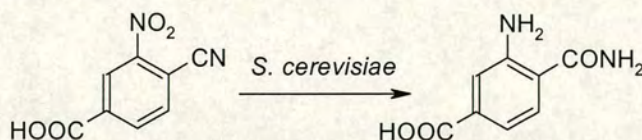
3-Amino-phthalamic acid **381**.

According to General Procedure V, 2-cyano-3-nitro-benzoic acid **377** (57.6 mg, 0.3 mmol), *S. cerevisiae* (4.50 g) and water (18 mL) were incubated for 24 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 3-amino-phthalamic acid **381** as a yellow solid (11.9 mg, 22.1 %).

$R_t$  (MeCN:H<sub>2</sub>O, 20:80) 6.3 min; Mp 171.1-173.2 °C; Data as in **381**.

4-Amino-isophthalamic acid **382**.

According to General Procedure V, 3-cyano-4-nitro-benzoic acid **378** (57.6 mg, 0.3 mmol), *S. cerevisiae* (4.50 g) and water (18 mL) were incubated for 4 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 4-amino-isophthalamic acid **382** as an orange solid (62.5 mg, 75.0 %); Data as in **382**.

3-Amino-terephthalamic acid **383**.

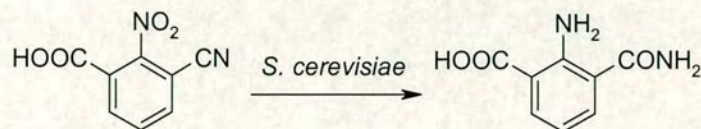
According to General Procedure V, 4-cyano-3-nitro-benzoic acid **379** (96.0 mg, 0.5 mmol), *S. cerevisiae* (7.50 g) and water (30 mL) were incubated for 3.5 h. After



work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 3-amino-terephthalamic acid **383** as an orange solid (61.2 mg, 73.4 %).

$R_t$  (MeCN:H<sub>2</sub>O, 15:85) 6.2 min; Mp 281.0-282.3 °C; Data as in **383**.

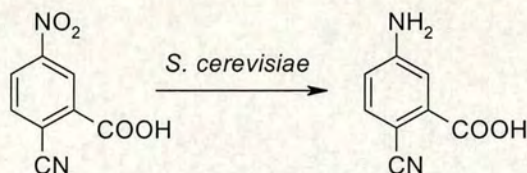
### 2-Amino-isophthalamic acid **391**.



According to General Procedure V, 3-cyano-2-nitro-benzoic acid **386** (57.6 mg, 0.3 mmol), *S. cerevisiae* (4.00 g) and water (16 mL) were incubated for 2 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 2-amino-isophthalamic acid **391** as a yellow gum (36.2 mg, 80.4 %).

$R_t$  (MeCN:H<sub>2</sub>O, 15:85) 3.4 min; Data as in **391**.

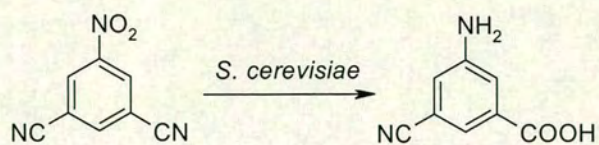
### 3-Amino-6-cyano-benzoic acid **392**.



According to General Procedure V, 6-cyano-3-nitro-benzoic acid **387** (96.0 mg, 0.5 mmol), *S. cerevisiae* (7.50 g) and water (30 mL) were incubated for 6 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 3-amino-6-cyano-benzoic acid **392** as a pale orange solid (50.3 mg, 60.3 %).

$R_t$  (MeCN:H<sub>2</sub>O, 20:80) 8.6 min; Mp 162.0-163.0 °C; Data as in **392**.



**3-Amino-5-cyano-benzoic acid 393.**

According to General Procedure V, 5-cyano-3-nitro-benzoic acid **389** (96.0 mg, 0.5 mmol), *S. cerevisiae* (7.50 g) and water (30 mL) were incubated for 8 h. After work-up, purification by preparative HPLC (acetonitrile:water, 10:90) afforded 3-amino-5-cyano-benzoic acid **393** as an orange solid (126.0 mg, 61.4%).

R<sub>t</sub> (MeCN:H<sub>2</sub>O, 10:90) 6.0 min; Mp 172.1 °C; Data as in **393**.



## 8. Bibliography.

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